Exhibit 1

UNIVERSITY of MARYLAND
SCHOOL OF PHARMACY

FENGTIAN XUE

Back to PSC Faculty

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Publications

Research

Group Members

Lab Pictures

Group News

Fengtian Xue, PhD, is an Associate Professor in the Department of Pharmaceutical Sciences at the University of Maryland School of Pharmacy

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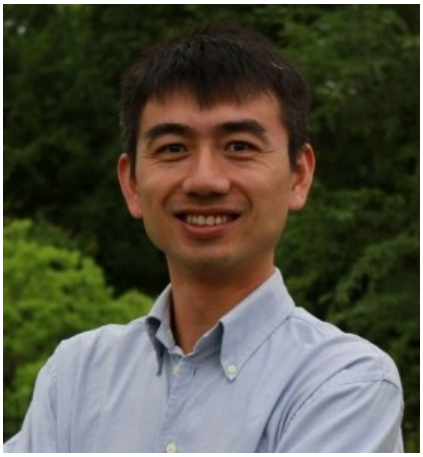

Postdoctoral – Accepting applicants
PhD – Currently recruiting for Fall 2022
MS – Currently recruiting for Fall 2022

Education

Postdoctoral, Medicinal Chemistry, Northwestern University

PhD, Chemistry, Brown University

BS, Chemistry, University of Science and Technology of China

Research Interests

Pre-clinical development of small molecule therapeutics for bacterial infections, alcohol use disorders, neurodegenerative diseases, and cancer

Current Projects (view)

Publications (view)

Exhibits 2-6, 11-13, 16, 19, 22-23, & 25

ZHP has designated the above exhibits as confidential. Plaintiffs are in the process of meeting and conferring with ZHP on the propriety of these designations. In accordance with the Court's Confidentiality and Protective order, Plaintiffs will forward the Exhibits to the Court directly via email for its in camera review.

Exhibit 7

Theoretical Investigation of *N*-Nitrosodimethylamine Formation from Nitrosation of Trimethylamine

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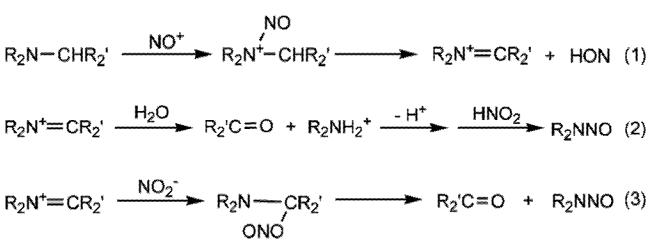
Tertiary amines have been demonstrated to be capable of undergoing nitrosative cleavage to produce carcinogenic *N*-nitrosamines. The reaction mechanism of the nitrosation of trimethylamine (TMA) to produce *N*-nitrosodimethylamine (NDMA) was investigated at the CBS-QB3 level of theory. The formation of NDMA from TMA was proposed to be initiated by the formation of an iminium ion, $Me_2N^+{=}CH_2$. Two different mechanisms (NOH elimination mechanism and oxidation abstraction mechanism) leading to $Me_2N^+{=}CH_2$ were investigated, and the oxidation abstraction mechanism was found to be mainly operative. This result indicates that the oxidation abstraction mechanism plays an important role in the nitrosation of both *N*,*N*-dialkyl aromatic and tertiary aliphatic amines. Starting from the iminium ion, two experimentally proposed mechanisms (pathways 1 and 2) and one new mechanism (pathway 3) were examined. Pathway 1 proposes that the iminium ion undergoes hydrolysis to give dimethylamine (DMA), which then can be further nitrosated to NDMA; pathway 2 proposes that the iminium ion reacts with NO_2^- and forms a neutral intermediate, which then collapses to NDMA. In pathway 3, the iminium ion reacts with N_2O_3 to give NDMA. Calculation results indicate that in aqueous solution pathway 1 is more feasible than pathways 2 and 3; moreover, the transformation from the iminium ion to NDMA is the rate-determining step. This work will be helpful to elucidate the formation mechanisms of the carcinogenic *N*-nitrosamines from the nitrosation of tertiary amines.

1. Introduction

It is well known that *N*-nitrosamines are a class of undesired industrial and environmental pollutants, many of which are carcinogenic, mutagenic, and teratogenic.[1−7] In particular, *N*-nitrosodimethylamine (NDMA), which is the simplest dialkylnitrosamine, has been demonstrated to be a potent carcinogen to various organs in animals, including liver, lung, and kidney.[8−12] As reported, NDMA has been found in air, soil, water, food, cosmetics, rubber products, and many other materials.[13−25] Therefore, the U.S. Environmental Protection Agency (U.S. EPA) defined NDMA as a probable human carcinogen.[26]

Because dialkylnitrosamines are of great interest in carcinogenesis, much attention has been focused on their formation mechanism, especially from secondary amines. Consequently, NDMA is generally believed to be formed from the reactions of dimethylamine (DMA) and nitrosating agents, such as N_2O_3, N_2O_4, and ONCl.[27−31] In addition to secondary amines, however, a wide variety of tertiary amines have also been demonstrated to react with nitrous acid to produce *N*-nitrosamines in aqueous solution.[32−44] In view of the ubiquity of tertiary amines, it is significant to understand the formation mechanism of *N*-nitrosamines from them, especially the formation of NDMA.

Previous experimental studies revealed that the nitrosation of tertiary amines proceeds via complex mechanisms depending on the structure of the amine, molar ratio of amine to nitrite, temperature, and pH.[34,35,38,42] Different from the nitrosation of secondary amines, it has been found that a relatively weak acid solution and mild warming are generally required for the *N*-nitrosamines formation from tertiary amines.[33,34,45] In addition, an α hydrogen atom is also required to exist in tertiary amine for the reaction to proceed.[33] On the basis of experimental results, the reactions were presumed to take place through the elimination of NOH from a nitrosammonium ion to produce an iminium ion $R_2N^+{=}CR_2'$, which then undergoes hydrolysis and further nitrosation (eqs 1 and 2). On the basis of this hypothesis, a viable alternative was then advanced as eq 3, implying the possibility of nucleophilic attack of NO_2^- toward the iminium ion to give the *N*-nitrosamine. Noticeably, the mechanisms described in eqs 1−3 became predominant in subsequent studies on the *N*-nitrosamines formation from normal aliphatic tertiary amines.



Several experimental studies of the reaction of simple aliphatic tertiary amines and nitrite obtained the similar result that the optimum pH for the production of the corresponding *N*-nitrosamines at elevated temperature (100 °C) is about 3.0 to 3.3.[37,38,41,46] It is noteworthy that the formation of nitrosating agent N_2O_3 is facilitated at this acidity. This suggests that a new mechanism rather than the mechanisms proposed as eqs 1−3 might become operative. A kinetic study performed by Ohshima and Kawabata[46] found that the rate of NDMA formation from TMA at pH 3.0 and 100 °C is proportional to the square of nitrite concentration. Therefore, a mechanism similar to eqs 1 and 2 was proposed, where the nitrosating agent

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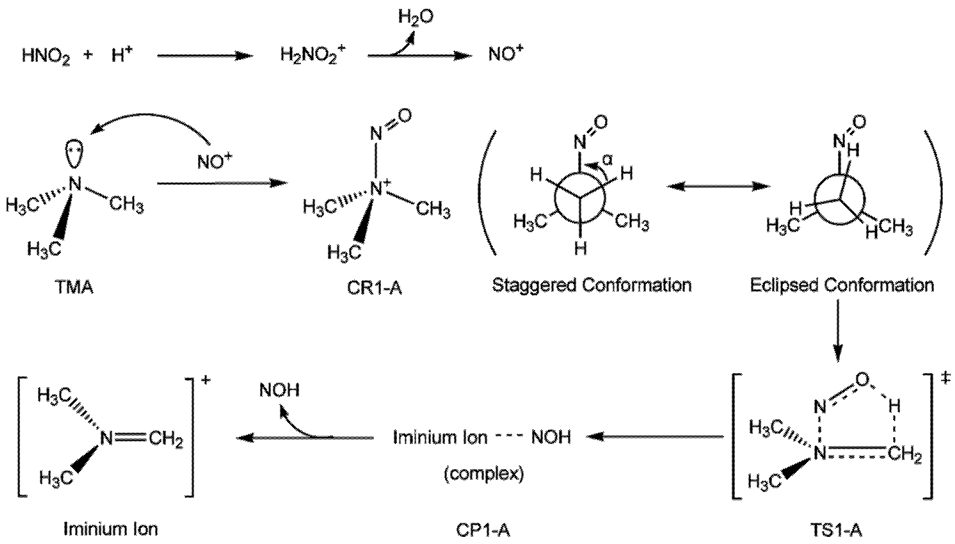
SCHEME 1: Mechanistic Pathway for the Formation of Iminium Ion with the NOH Elimination Mechanism



was suggested to be N_2O_3 rather than HNO_2. This hypothesis is reasonable because N_2O_3 has been demonstrated to be a more potent nitrosating agent than HNO_2 in the nitrosation of secondary amines.[30] However, the possibility for the direct nitrosation of the iminium ion by N_2O_3 has not yet been taken into consideration.

In addition, two recent studies[47,48] suggested that the tertiary amine may be an important precursor of carcinogenic NDMA during water disinfection process, and the proposed mechanism also involves the dealkylation of tertiary amine, which is similar to the proposed nitrosation mechanism of tertiary amines. Therefore, the investigation of the nitrosation mechanism of tertiary amines may also provide a useful model to investigate the newly found reaction, which may take place during water treatment.

Few theoretical investigations were found to make contributions to elucidate the reaction mechanism of nitrosation of tertiary amines. To better understand this kind of reaction, the present work is a theoretical research on the detailed mechanistic pathways for the formation of *N*-nitrosamine from the reaction of tertiary amine with nitrite. Trimethylamine (TMA), the simplest trialkylamine and one that can be derived from the ordinary diet,[49−51] has been suggested to be a possible precursor of NDMA,[38,46,52] and so it was selected as the model compound.

2. Theoretical Methods

All structures of reactants, transition states, intermediates, and products were fully optimized by using the B3LYP method (Becke's three-parameter nonlocal exchange functional[53] with the correlation functional of Lee, Yang, and Parr[54]) in conjunction with 6-311+G(d,p) basis set. Vibrational frequencies were also calculated at the same level to characterize the nature of each stationary point. The intrinsic reaction coordinate (IRC)[55] calculations were performed to confirm that every transition state connects the corresponding reactant and product through the minimized-energy pathway. On the basis of the optimized geometries at the B3LYP/6-311+G(d,p) level, reoptimizations of these stationary points were performed with the complete basis set (CBS-QB3) methodology, in which B3LYP density functional theory is combined with the CBSB7 basis set.[56]

Because the proposed mechanisms are expected to take place in aqueous solution, the solvent effect of water on the reactions of NDMA formation from TMA was also taken into account in this study. On the basis of the optimized geometries obtained at the CBS-QB3 level, the single-point energy calculation was carried out by using the conductorlike polarizable continuum model (CPCM)[57] at the CCSD(T)/6-311+G(d,p) level,[58] denoted as CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7. According to the calculation results from Takano and Houk,[59] the UAKS cavity was used in this study to evaluate the aqueous solvation effects, and the rest of the parameters of the models have been kept as default values from the Gaussian 03 program package.[60] Because the improved density functional PBE1W has been proven to be better than B3LYP method for calculating the energies of water clusters,[61,62] we also investigated the hydrolysis mechanism by using the PBE1W method, and the result will be used to compare with that at the CBS-QB3 level. The absolute energy data are provided in the Supporting Information. All calculations presented in this work were carried out with the Gaussian 03 program package.

3. Results and Discussion

3.1. Formation of the Iminium Ion. Two different pathways were investigated to elucidate the formation of the iminium ion. The first pathway is the mechanism described in eq 1, which involves the formation of a nitrosammonium ion Me_3NNO^+, followed by elimination of NOH to give the iminium ion (NOH elimination mechanism). The second pathway is a new mechanism recently proposed by Loeppky et al.[63] This mechanism involves the oxidation of the tertiary amines to a radical cation by NO^+, followed by the H-abstraction to produce the iminium ion by nitrogen dioxide, NO_2 (oxidation abstraction mechanism). The two mechanisms will be discussed in turn.

3.1.1. NOH Elimination Mechanism. The NOH elimination mechanism involves the formation of a nitrosammonium ion (Me_3NNO^+) and followed by elimination of NOH to give the iminium ion. The detailed reaction pathway is illustrated in Scheme 1, and the fully optimized structures of all stationary points involved in this process are shown in Figure 1. The relative energies are listed in Table 1.

As shown in Scheme 1, TMA first undergoes electrophilic attack of NO^+ to form a nitrosammonium ion, which is a positively charged reactant complex (CR1-A). Figure 1 shows that the N−N bond length and the angle ∠O−N−N in CR1-A were predicted to be 1.884 Å and 111.9°, respectively. As listed in Table 1, the enthalpy change (ΔH) was calculated to be

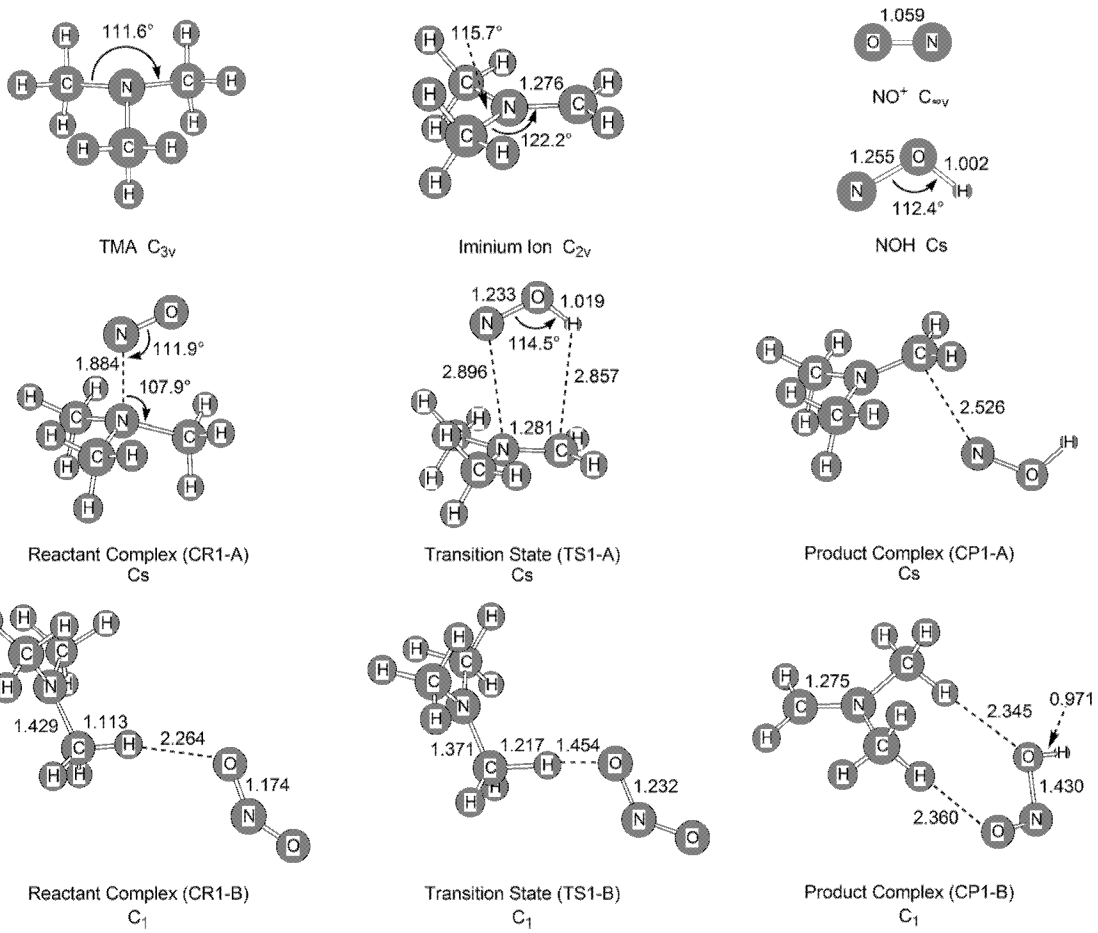


Figure 1. Optimized geometries and main parameters of all stationary points involved in the process of the formation of the iminium ion (distances in angstroms).

TABLE 1: Relative Energies (RE), Enthalpies (RH), and Free Energies (RG) of each Stationary Point Involved in the Formation of the Iminium Ion at the CBS-QB3 Level in the Gas Phase and Aqueous Solution[a,b]

species	RE	RH	RG	RE_w[b]
NOH elimination mechanism				
TMA + NO^+	0.00	0.00	0.00	0.00
CR1-A	−58.64	−58.97	−50.33	−20.65
TS1-A	−3.80	−3.96	3.94	32.97
CP1-A	−10.46	−10.14	−3.85	27.66
iminium ion + NOH	−2.20	−1.84	−3.64	29.88
oxidation abstraction mechanism				
TMA^+ + NO_2	0.00	0.00	0.00	0.00
CR1-B	−27.71	−27.19	−20.05	48.53
TS1-B	17.26	16.78	27.32	39.02
CP1-B	−47.96	−47.98	−39.08	−46.12
iminium ion + HNO_2	−40.83	−41.38	−39.30	−45.24

[a] Gas phase: relative energies, enthalpies, and free energies in kilocalories per mole. [b] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level for comparison.

−58.97 kcal/mol, which indicates that the formation of CR1-A is an exothermic process in the gas phase. The change of Gibbs free energies (ΔG) was calculated to be −50.33 kcal/mol, indicating that the formation of CR1-A from TMA and NO^+ is thermodynamically favored and can take place spontaneously at 298 K and 1 atm. Then, a cis elimination of NO moiety with a hydrogen atom from an α-carbon in CR1-A occurs to produce the NOH and iminium ion. Reaction barrier for this elimination was predicted to be 54.84 kcal/mol in the gas phase.

Moreover, Scheme 1 shows that two steps are required for the formation of iminium ion. The first step is the transformation of CR1-A from a staggered conformation to an eclipsed one, which was caused by the rotation of N−C single bond. To characterize the nature of this transformation, an additional calculation of potential energy surface (PES) scan was performed at the B3LYP/CBSB7 level, and the result was illustrated in Figure 2. As shown in the PES, the energy continually increases when changing from the staggered conformation to the eclipsed one, and the energy gap between the two conformations is only 1.51 kcal/mol at the CBS-QB3 level, which indicates that the transformation occurs easily. It is interesting to note that the eclipsed conformation of CR1-A per se is not a stationary point but a transition state on the PES because the imaginary frequency is 155.2i cm^{-1}. The vibrational mode of this imaginary frequency corresponds to a rocking vibration of the methyl group through the rotation of the N−C single bond and connects two identical staggered conformations. In the second step, a five-membered cyclic transition state (TS1-A, 137.7i cm^{-1}) is formed from CR1-A in the eclipsed conformation. As illustrated in Figure 1, the N−N and H−C distances in TS1-A were calculated to be 2.896 and 2.857 Å, respectively. These unexpectedly long distances were possibly caused by the fact that the positively charged TS1-A is an electron deficient species. In addition, only slight differences were found between the geometries of NOH moiety in TS1-A and the fully optimized NOH molecule with C_s point group, as shown in Figure 1. A

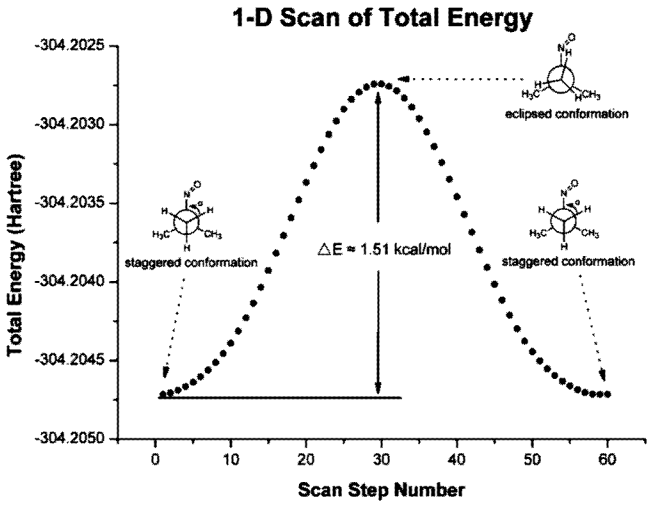


Figure 2. Scan plot of potential energy surface (PES) for the transformation of CR1-A from the staggered conformation to the eclipsed conformation at the B3LYP/CBSB7 level.

similar situation can also be observed between the other moiety of TS1-A and the fully optimized iminium ion. Both of the vibrational mode of imaginary frequency and IRC calculations at the B3LYP/6-311+G(d,p) level confirm that this transition state does connect the corresponding reactant complex (CR1-A) and product complex (CP1-A). The energy required to separate the NOH and iminium ion from CP1-A was calculated to be 8.26 kcal/mol, as shown in Table 1.

3.1.2. Oxidation Abstraction Mechanism. The oxidation abstraction mechanism involves the oxidation of the TMA to a radical cation by NO^+ and then followed by the H-abstraction to produce the iminium ion by NO_2. The detailed reaction pathway of this mechanism is illustrated in Scheme 2, and the fully optimized structures of all stationary points involved in this mechanism are shown in Figure 1. The relative energies are listed in Table 1.

As shown in Scheme 2, similar as the case of Scheme 1, TMA first undergoes electrophilic attack of NO^+ to form the nitrosammonium ion (CR1-A). This is followed by the elimination of nitric oxide NO to produce a radical cation, TMA^+. The TMA^+ further undergoes the H-abstraction by the attack of NO_2 to form the iminium ion and HNO_2. The origin of the radical NO_2 is described as eqs 4−6. Two HNO_2 molecules first react to yield N_2O_3, followed by the homolytic dissociation of the N−N bond in N_2O_3 to give NO_2.

$$H^+ + NO_2^- \rightleftharpoons HNO_2 \quad (4)$$

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad (5)$$

$$N_2O_3 \rightleftharpoons {}^{\cdot}NO + {}^{\cdot}NO_2 \quad (6)$$

The total energy of the TMA^+ and NO was calculated to be remarkably lower than that of the TMA and NO^+ by 33.51 kcal/mol. This result implies that the oxidation of TMA to TMA^+ by NO^+ is exothermic. It is illustrated in Figure 1 that the main geometrical change for the reaction of TMA^+ and NO_2 is the transfer of a hydrogen atom from the TMA^+ to NO_2. It is noteworthy that the energy barrier of the oxidation abstraction mechanism in the gas phase was calculated to be 44.97 kcal/mol, which is lower than the barrier of the NOH elimination mechanism (54.84 kcal/mol), as shown in Table 1.

It should be noted that both the NOH elimination and oxidation abstraction mechanisms proposed the positively charged NO^+ as the oxidative species in this article. However, there is still the possibility of the other species such as NO_2 or N_2O_3 as the oxidant. Therefore, what is the oxidative species to amines may be still an open question.

3.2. Formation of NDMA from Iminium Ion. Generally, there are two main reaction mechanisms for the formation of NDMA from the iminium ion. In the first mechanism, the iminium ion undergoes hydrolysis to be degraded to a secondary amine DMA, which can then be further nitrosated to NDMA. In the second mechanism, the iminium ion directly reacts with nitrosating species (NO_2^- or N_2O_3) to produce NDMA. In this study, three pathways were considered. Pathway 1 belongs to the first mechanism, in which the iminium ion undergoes hydrolysis to form DMA. Pathways 2 and 3 belong to the second mechanism, in which the iminium ion undergoes a nucleophilic attack from NO_2^- and N_2O_3, respectively, and then collapses to NDMA. The former two pathways were proposed on the basis of previous experimental results, and the third is a pathway that was put forward on the basis of the fact that N_2O_3 can be formed under the mild acid condition. Detailed discussions for pathways 1, 2, and 3 will be given in turn.

3.2.1. Reaction Pathway 1: Hydrolysis Mechanism. Pathway 1 proposes that the nascent iminium ion undergoes hydrolysis to give a protonated adduct, which then loses a H^+ to give a formaldehyde molecule and DMA, which will be nitrosated finally to NDMA. Two different hydrolysis mechanisms were examined: (A) non-assisted hydrolysis mechanism (only one H_2O molecule involved) and (B) water-assisted hydrolysis mechanism (two H_2O molecules involved). Because the improved density functional PBE1W has been proven to be better than the B3LYP method for calculating the energies of water clusters,[60,61] the hydrolysis mechanism was also investigated by using the PBE1W method, and the result will be used to compare with that at the CBS-QB3 level (Tables 2 and 3). The detailed reaction pathways are illustrated in Schemes 3 and 4. The hybridization change of N and C atoms involved in the mechanism is described in Figure 3, and the fully optimized structures of all stationary points involved in this mechanism are shown in Figures 4 and 5. The relative energies are listed in Tables 2 and 3.

A. Non-Assisted Hydrolysis Mechanism. As illustrated in Scheme 3, a reactant complex (CR2-A) composed of the nascent iminium ion and one H_2O molecule is first formed. Table 2 shows that the enthalpy change ΔH for the formation of CR2-A was calculated as −9.21 kcal/mol, which predicts an exothermic process. As illustrated in Figure 3, in CR2-A, the N and C atoms are sp^2 hybridized, and the p orbitals of the N and C atoms that are perpendicular to the planar iminium ion overlap each other and form a stable conjugated system. The iminium ion moiety shows a planar structure in which the dihedral angles D(C−N−C−C) and D(H−C−H−N) are 178.5 and 178.7° (Figure 4), respectively.

Further approach of the iminium ion and H_2O leads to the formation of a four-membered cyclic transition state (TS2-A, 1374.1i cm^{-1}). In this process, as shown in Figure 4, the O−C bond length decreases from 2.549 to 1.513 Å, whereas the distance between H and N atoms reduces from 3.786 to 1.478 Å, which indicates the formation of C−O and N−H bonds. H−O and N−C bond lengths are increased by 0.182 and 0.177 Å, respectively, which indicates the cleavage of these two bonds. The dihedral angles of D(C−N−C−C) and D(H−C−H−N) reduce to 134.8 and 135.2° when changing from CR2-A to TS2-A, respectively, which implies that the original sp^2 hybridized N and C atoms in CR2-A are going to convert to sp^3

SCHEME 2: Mechanistic Pathway for the Formation of Iminium Ion with the Oxidation Abstraction Mechanism

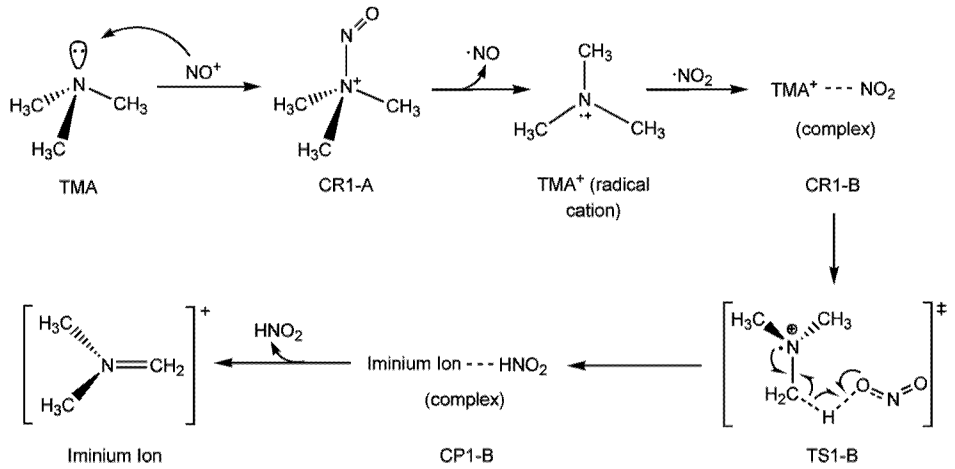


TABLE 2: Relative Energies (RE), Enthalpies (RH), and Free Energies (RG) of Each Stationary Point Involved in the Non-Assisted Hydrolysis Mechanism at the CBS-QB3 Level in the Gas Phase and Aqueous Solution[a,b,c]

species	RE	RH	RG	RE_w[b]	RE_w[c]
iminium ion + H_2O	0.00	0.00	0.00	0.00	0.00
CR2-A	−9.33	−9.21	−3.01	−3.21	−3.24
TS2-A	30.88	29.20	40.34	29.63	29.39
CP2-A	−6.99	−8.24	1.68	−14.42	−16.28

[a] Gas phase: relative energies, enthalpies, and free energies in kilocalories per mole. [b] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level for comparison. [c] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//PBE1W/CBSB7 level for comparison.

TABLE 3: Relative Energies (RE), Enthalpies (RH), and Free Energies (RG) of Each Stationary Point Involved in the Water-Assisted Hydrolysis Mechanism at the CBS-QB3 Level in the Gas Phase and Aqueous Solution[a,b,c]

species	RE	RH	RG	RE_w[b]	RE_w[c]
iminium ion + $2H_2O$	0.00	0.00	0.00	0.00	0.00
CR2-B	−17.44	−18.22	−1.87	−6.83	−5.16
TS2-B	5.97	3.31	24.64	8.47	8.24
CP2-B	−21.90	−23.52	−4.86	−19.36	−19.18

[a] Gas phase: relative energies, enthalpies, and free energies in kilocalories per mole. [b] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level for comparison. [c] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//PBE1W/CBSB7 level for comparison.

hybridization in the process, as illustrated in Figure 3. The conversion of hybridization makes the N and C atoms more capable of accepting the H and O atoms of the H_2O molecule, respectively. In fact, the hybridization of N and C atoms in TS2-A is more like a middle state between sp^2 and sp^3, and further decreased D(C−N−C−C) and D(H−C−H−N) can be observed in the product complex (CP2-A) when compared with that of TS2-A (Figure 4).

As shown in Table 2, the energy barrier for this reaction was calculated to be 40.21 kcal/mol in the gas phase. This relatively high barrier can be rationalized by the stable CR2-A and the high-lying four-membered cyclic transition state TS2-A with strong ring strain.

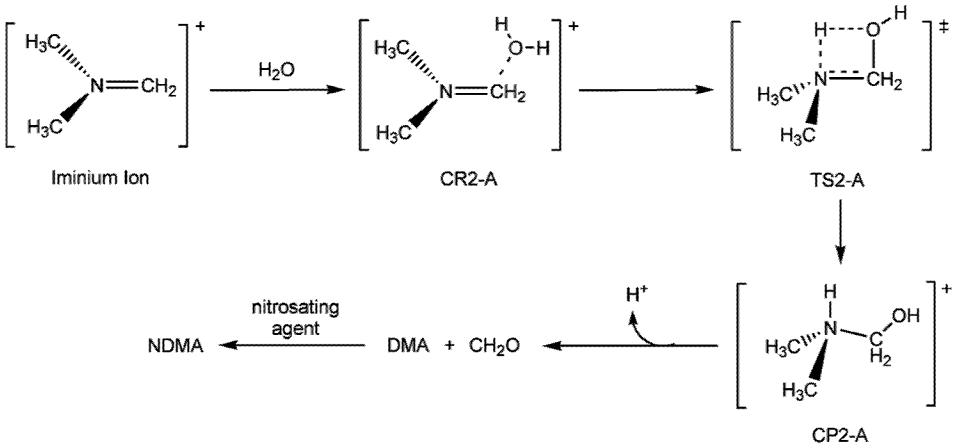
Natural bond orbital (NBO) analysis[64] for CP2-A showed that the highest positively charged position locates at the hydrogen atom H (+0.500 e) adjacent to oxygen atom. Therefore, this hydrogen atom might be expected to easily undergo the attack of nucleophiles in solution. Consequently, the loss of a proton H^+ will give rise to the formaldehyde and DMA, which can be further nitrosated to NDMA by a nitrosating agent, as shown in Scheme 3.

B. Water-Assisted Hydrolysis Mechanism. Two H_2O molecules are required for the water-assisted hydrolysis mechanism in Scheme 4, which is different from the non-assisted hydrolysis mechanism. It is shown that a reactant complex (CR2-B) between the nascent iminium ion and two H_2O molecules is first formed. As shown in Table 3, enthalpy change ΔH for the formation of CR2-B was calculated to be −18.22 kcal/mol, which predicts that this formation is an exothermic process in the gas phase. Similar to the case of CR2-A, in Figure 5, the N and C atoms in CR2-B are sp^2 hybridized because the iminium ion moiety shows a planar structure in which the dihedral angles D(C−N−C−C) and D(H−C−H−N) are 173.7 and 174.1°, respectively. As discussed in the non-assisted hydrolysis mechanism, the planar structure indicates the formation of an intramolecular stable conjugated system.

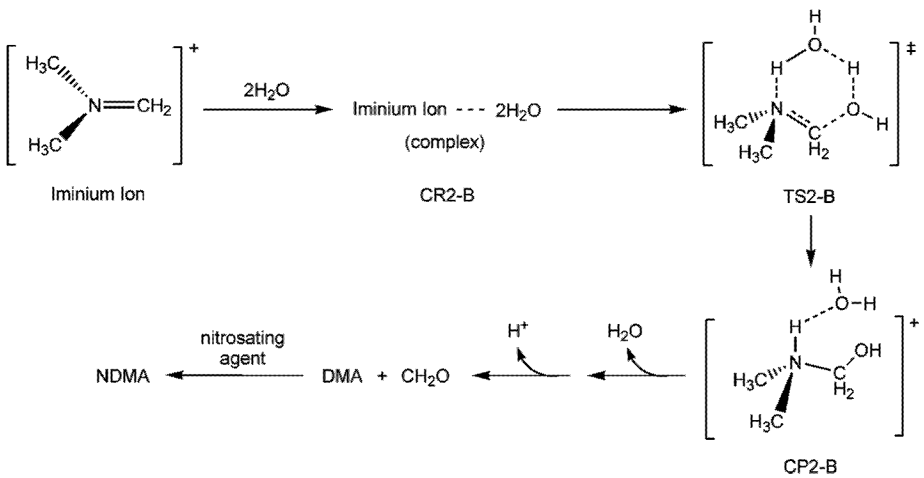
As shown in Scheme 4, further approach of the iminium ion and H_2O molecules in CR2-B leads to the formation of a six-membered cyclic transition state (TS2-B, 276.5i cm^{-1}). In this process, the O−C bond length decreases from 2.372 to 1.503 Å, whereas the N−C bond length increases from 1.281 to 1.427 Å. The dihedral angles of D(C−N−C−C) and D(H−C−H−N) reduce to 128.5 and 129.8° when changing from CR2-B to TS2-B, respectively, which implies that the original sp^2 hybridized N and C atoms in CR2-B are going to convert to sp^3 hybridization in the process, as illustrated in Figure 5. Further changes following these tendencies give rise to the product complex (CP2-B), in which the O−C bond length decreases to 1.384 Å, whereas the N−C bond length increases to 1.525 Å, and the dihedral angles of D(C−N−C−C) and D(H−C−H−N) reduce to 127.4 and 115.8°, respectively. Similar to the case of Scheme 3, Scheme 4 shows that the loss of a water molecule and a proton H^+ from the nascent CP2-B gives the formaldehyde and DMA, which can be further nitrosated to NDMA by a nitrosating agent. The nitrosation of secondary amines has already been extensively studied,[27−31] and the DMA has been confirmed to be easily nitrosated into NDMA in an acidic nitrite solution.

As shown in Table 3, the energy barrier for water-assisted hydrolysis mechanism was calculated to be 23.41 kcal/mol in the gas phase. It is notable that this barrier is almost half the magnitude of the barrier for the non-assisted hydrolysis mech-

SCHEME 3: Mechanistic Pathway for the Formation of NDMA with the Non-Assisted Hydrolysis Mechanism of Pathway 1



SCHEME 4: Mechanistic Pathway for the Formation of NDMA with the Water-Assisted Hydrolysis Mechanism of Pathway 1



anism (40.21 kcal/mol). This lower energy barrier can be rationalized by the fact that the transition state TS2-B, composed of a six-membered ring, is much more stable than the high-lying four-membered cyclic transition state TS2-A.

In addition to the hydrolysis mechanisms discussed above, the oxygen atom of water molecule may also attack the iminium ion to the nitrogen atom to form two different transition states (TS2-A′ and TS2-B′). These possibilities were also examined; however, the corresponding energy barriers were predicted to be high as 77.78 and 62.93 kcal/mol at the PBE1W/CBSB7 level in the gas phase, respectively. Therefore, they are not expected to occur. The absolute energies were collected in the Supporting Information (Tables S13 and S14).

3.2.2. Reaction Pathway 2: Nucleophilic Attack of NO_2^-. The iminium ion, which is known to be highly reactive toward nucleophiles,[65,66] could therefore be expected to easily undergo the nucleophilic attack by free nitrite (NO_2^-) to give a neutral prereactant (designated as PR3). This prereactant then directly collapses to formaldehyde and NDMA. The detailed reaction pathway is illustrated in Scheme 5, and the fully optimized structures of all stationary points involved in this pathway are collected in Figure 6. The relative energies are listed in Table 4.

As shown in Scheme 5, after the formation of PR3, the movement of electrons may occur to give a transition state (TS3, 383.5i cm^{-1}). It can be found that TS3 is not a real four-

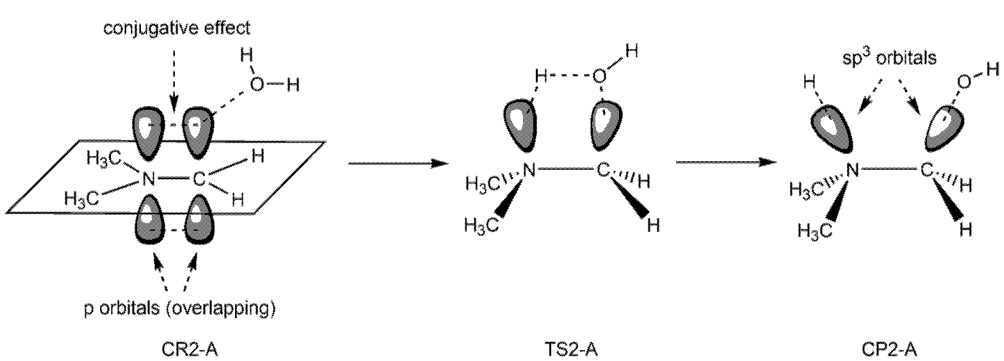


Figure 3. Schematic profile for the conversion of hybridization of N and C atoms involved in the non-assisted hydrolysis mechanism of pathway 1.

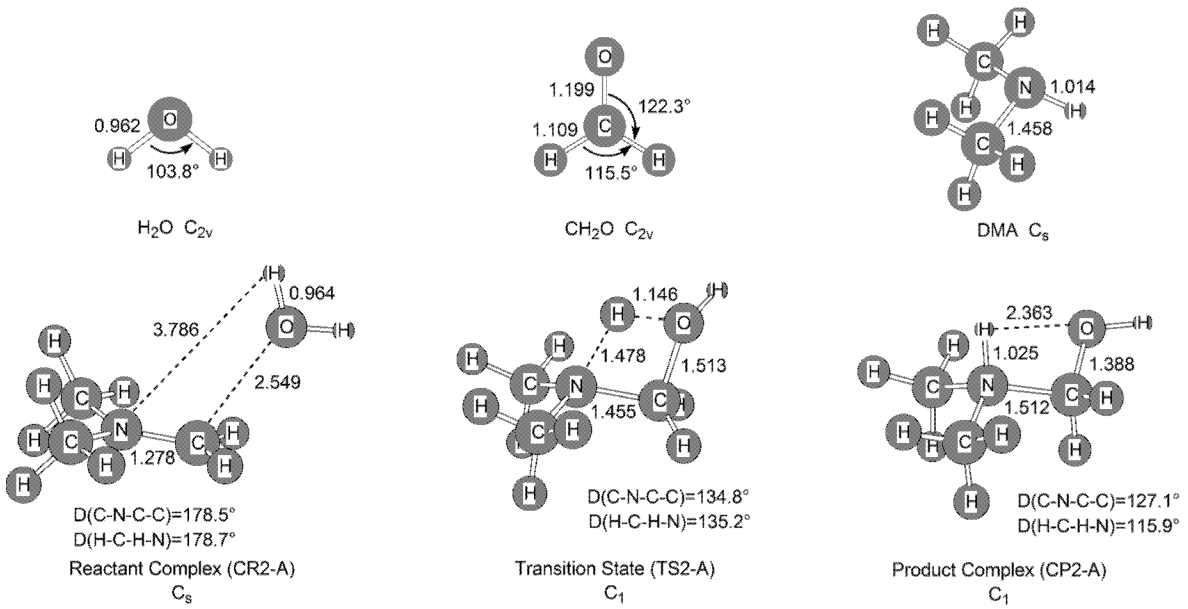


Figure 4. Optimized geometries and main parameters of all the stationary points involved in the non-assisted hydrolysis mechanism of pathway 1 (distances in angstroms, dihedral angle in degrees).

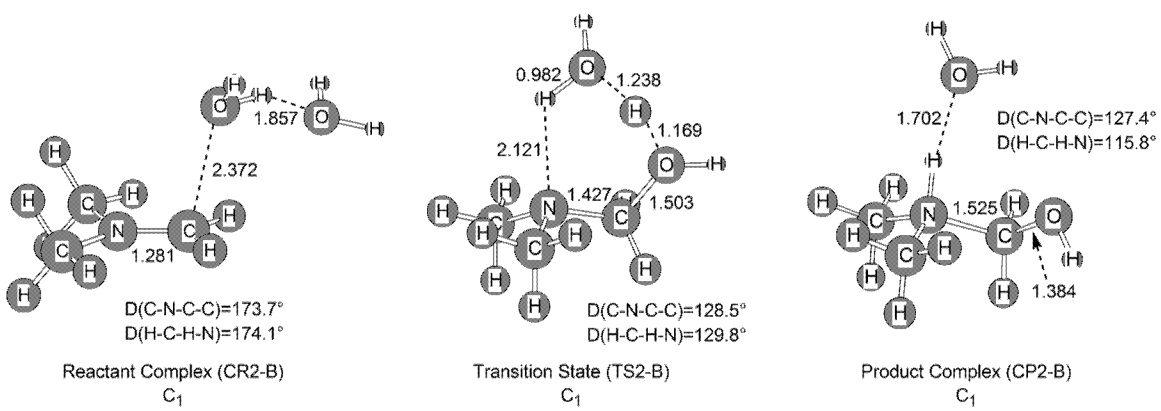
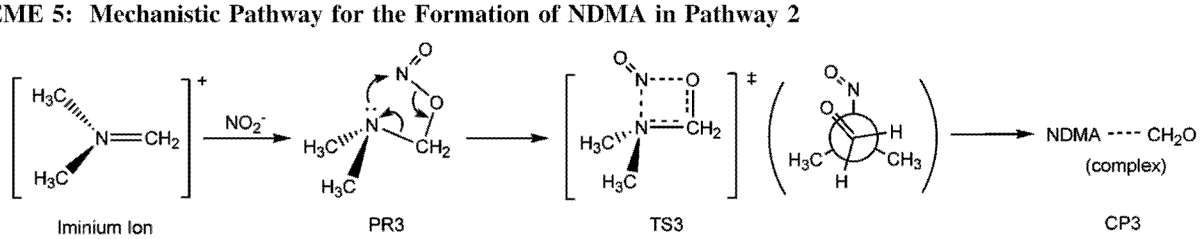


Figure 5. Optimized geometries and main parameters of all the stationary points involved in the water-assisted hydrolysis mechanism of pathway 1 (distances in angstroms, dihedral angle in degrees).

SCHEME 5: Mechanistic Pathway for the Formation of NDMA in Pathway 2



membered cyclic transition state. Newman projection in Scheme 5 implies that TS3 actually holds a conformation between the staggered and eclipsed one, and this is supported by the dihedral angle D(O−C−N−N) calculated to be 45.3°. (See Figure 6.) It can be rationalized by the fact that this conformation avoids strong ring strain and steric hindrance for stabilization. In the process from PR3 to TS3, the changes of bonds shown in Figure 6 indicate that the original N−C and N−O bonds are partially cleavaged, whereas the N−N bond is partially formed. The C−O bond length decreases from 1.430 to 1.257 Å, indicating that a double bond is formed. As shown in Table 4, in the gas phase, the energy barrier for the reaction was calculated to be 27.94 kcal/mol, which is close to that of the water-assisted hydrolysis mechanism in pathway 1 (23.41 kcal/mol).

In the process from TS3 to CP3, two types of geometric changes can be observed: the elongation of the distances of N−C and N−O bonds between CH_2O moiety and the other and the reduction of N−N and C−O bond lengths. In addition, the changes of the two moieties from their previous tetrahedral configuration to a planar configuration demonstrate that both of the N and C atoms change from sp^3 to sp^2 hybridization. Furthermore, changes of the geometry will form the product complex (CP3), in which the formaldehyde and NDMA are connected by two O−H hydrogen bonds, as shown in Figure 6.

3.2.3. Reaction Pathway 3: Direct Nitrosation by N_2O_3. As discussed previously, the optimum pH for the conversion of TMA to NDMA at elevated temperature has been proven experimentally to be about 3.0 to 3.3.[37,38,41,46] It is well known that within this pH range N_2O_3 can be easily formed. On the basis of these results, a new mechanism is proposed in which the iminium ion directly reacts with N_2O_3. The detailed reaction

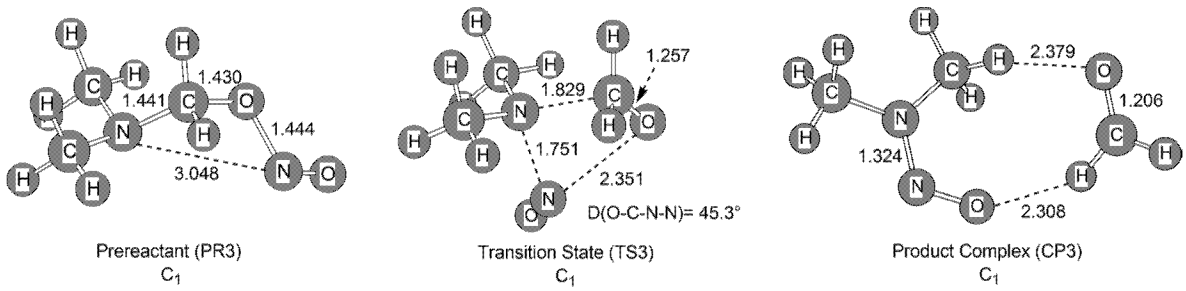


Figure 6. Optimized geometries and main parameters of all stationary points involved in pathway 2 (distances in angstroms, dihedral angle in degrees).

TABLE 4: Relative Energies (RE), Enthalpies (RH), and Free Energies (RG) of Each Stationary Point Involved in the Reaction of the Iminium Ion with NO_2^- at the CBS-QB3 Level in the Gas Phase and Aqueous Solution[a,b]

species	RE	RH	RG	RE_w[b]
iminium ion + NO_2^-	0.00	0.00	0.00	0.00
PR3	−128.18	−128.74	−117.58	−15.21
TS3	−100.24	−100.72	−89.27	10.25
CP3	−133.31	−132.58	−125.33	−18.95
CH_2O + NDMA	−129.67	−129.13	−130.45	−17.31

[a] Gas phase: relative energies, enthalpies, and free energies in kilocalories per mole. [b] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level for comparison.

pathway is illustrated in Scheme 6, and the fully optimized structures of all stationary points involved in this pathway are collected in Figure 7. The relative energies are listed in Table 5.

As shown in Scheme 6, two steps are required for this reaction. The first step is the nucleophilic attack of N_2O_3 to the iminium ion to produce a positively charged intermediate (IM), and the second step is the collapse of IM to give the final products: NDMA, formaldehyde, and NO^+. It is evident that NO^+ actually plays the role of catalyst in the whole reaction, and it may reparticipate in the formation of iminium ion. (See Scheme 1.)

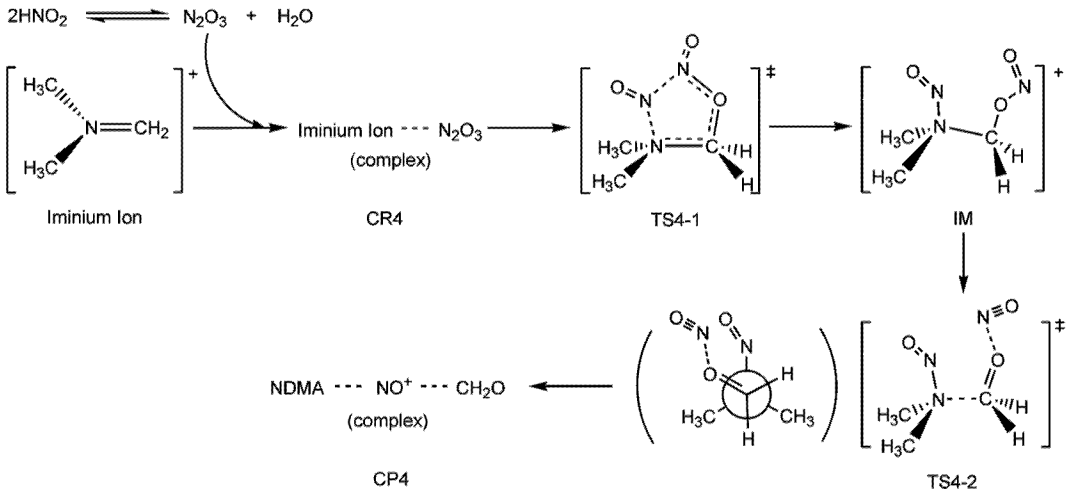
The nucleophilic attack of N_2O_3 to the iminium ion starts from a reactant complex CR4, followed by the formation of a five-membered cyclic transition state TS4−1 (345.5i cm^{-1}), which then further produces an intermediate IM. The energy barrier of this step was calculated to be 29.12 kcal/mol in the gas phase, and the enthalpy change ΔH shown in Table 5 indicates that the reaction is an exothermic process. IRC calculation along the reverse reaction pathway shows two types of geometric changes: the elongation of O−C and N−N bond lengths between the iminium ion and N_2O_3 moieties and the reduction of N−N bond length to be N_2O_3 and C−N bond length in iminium ion moiety. These geometric changes indicate that the energy 29.12 kcal/mol required for the transformation from CR4 to TS4−1 is mainly utilized to rotate and dissociate the N−N bond in N_2O_3 as well as elongate the C−N bond in the moiety of iminium ion. During this process, the dihedral angles D(H−C−N−H) and D(C−N−C−C) reduce by 40.8 and 31.9°, respectively. This indicates that the hybridization of the N and C atoms in the iminium ion moiety change from sp^2 to sp^3, which is similar to the case of hydrolysis mechanism (pathway 1, Figure 3). Further changes of the structure encounter an intermediate IM. It can be observed that the N−N bond in the original N_2O_3 moiety is totally separated in IM, and the original planar iminium ion moiety changes into a tetrahedron configuration as a result of the further reduction of corresponding dihedral angles.

In the second step of the reaction, TS4−2 (211.9i cm^{-1}) connects the nascent intermediate IM and the product complex CP4, as depicted in Scheme 6. The reaction barrier for this step was calculated as 15.31 kcal/mol, which is lower than the barrier (29.12 kcal/mol) of the first step reaction in the gas phase. This implies that the formation of the intermediate IM is the rate-determining step for pathway 3. Notably, the energy barrier (29.12 kcal/mol) of the first step is close to the barrier (23.41 kcal/mol) calculated for the water-assisted hydrolysis mechanism in pathway 1 and the barrier (27.94 kcal/mol) for pathway 2. In addition, it can be found that the products NDMA and formaldehyde have already formed in TS4−2, for which a staggered conformation can be observed. (See the Newman projection in Scheme 6.) The main vibrational mode for the imaginary frequency of TS4−2 corresponds to the stretching vibration of N−C bond, indicating that the energy requirement (15.31 kcal/mol) for overcoming the barrier is mainly utilized to dissociate the N−C bond. Additionally, N−O bond length was also found to be elongated from 1.638 to 1.895 Å when changing from IM to TS4−2. Also, the N−N and C−O bond lengths are decreased by 0.454 and 0.127 Å, respectively, predicting the formation of the two bonds. Further changes of the structure give rise to the product complex (CP4) composed of NDMA, formaldehyde, and NO^+. It is interesting to note that the changes of hybridization of the N and C atoms exhibit a reverse process relative to that of the first step in the reaction (formation of IM from CR4), for both of the two atoms changing back to sp^2 from sp^3 hybridization. This finding is supported by the changes of the corresponding dihedral angles shown in Figure 7.

3.3. Effects of Solvent. Because the attention of this article is focused on environmental nitrosamine formation, for which most reactions should be expected to occur in aqueous solution, the solvent effect of water on NDMA formation from TMA was taken into account. On the basis of the optimized geometries obtained at the CBS-QB3 level, the single-point energy calculation was carried out by the use of the conductorlike polarizable continuum model (CPCM) at the CCSD(T)/6-311+G(d,p) level, denoted as CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7. The corresponding relative energy data (RE_w) in water are presented in Tables 1−5 for comparison with those in the gas phase.

As for the formation of the iminium ion, data in Table 1 show that the energy barrier for the NOH elimination mechanism is 53.62 kcal/mol in aqueous solution. Significantly, the oxidation abstraction mechanism becomes a barrierless process with a negative energy barrier (−9.51 kcal/mol). Moreover, the oxidation abstraction mechanism is exothermic, and a considerable energy (45.24 kcal/mol) was predicted to be released from the

SCHEME 6: Mechanistic Pathway for the Formation of NDMA in Pathway 3



reaction. Therefore, the TMA^+ would be expected to react easily with NO_2 because of the low energy barrier. The results actually lead to a conclusion that the oxidation abstraction mechanism is more favored than the NOH elimination mechanism and is probably the one that is mainly operative to form iminium ion. Noticeably, this result supports the feasibility of the experimentally proposed oxidation abstraction mechanism.[63] Moreover, it also indicates that the newly proposed oxidation abstraction mechanism plays an important role not only in the nitrosation of *N*,*N*-dialkyl aromatic amines[63] but also in the case of tertiary aliphatic amines. Furthermore, because the formation of N_2O_3 and its cleavage product NO_2 (eqs 4−6) is facilitated

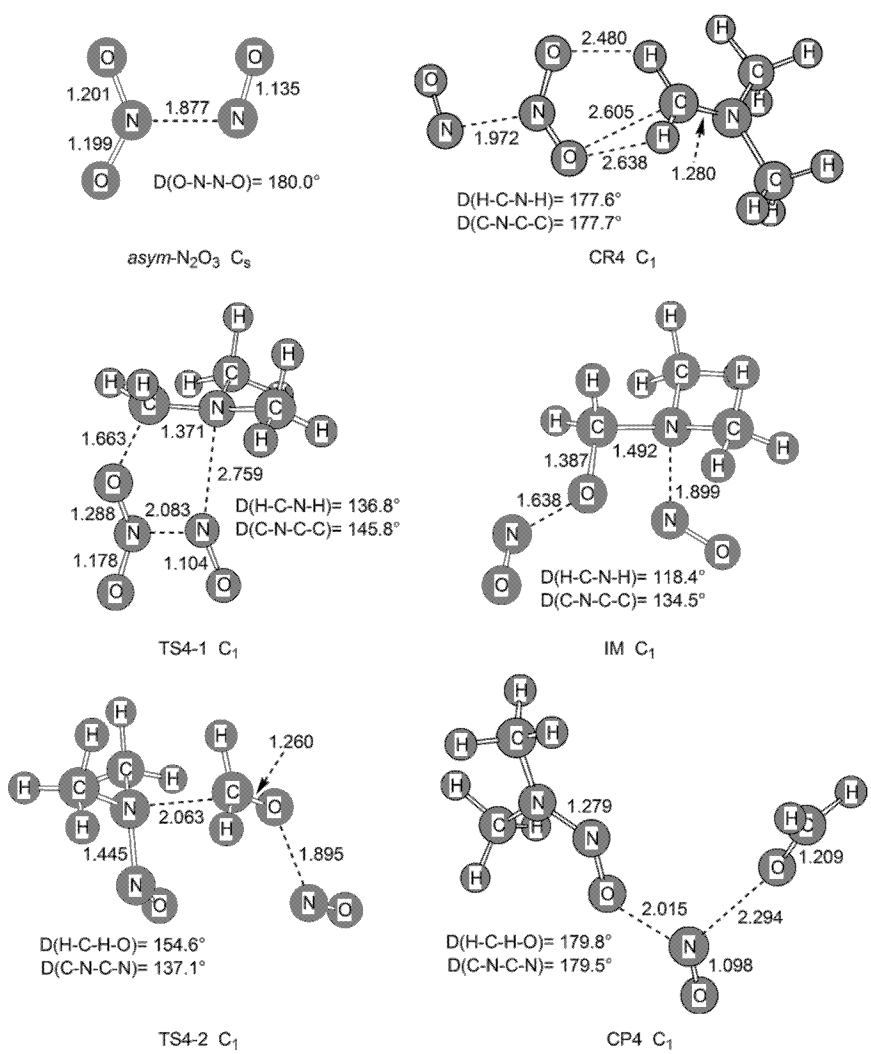


Figure 7. Optimized geometries and main parameters of all stationary points involved in pathway 3 (distances in angstroms, dihedral angle in degrees).

TABLE 5: Relative Energies (RE), Enthalpies (RH), and Free Energies (RG) of each Stationary Point Involved in the Reaction of the Iminium Ion with N_2O_3 at the CBS-QB3 level in the Gas Phase and Aqueous Solution[a,b]

species	RE	RH	RG	RE_w[b]
iminium ion + N_2O_3	0.00	0.00	0.00	0.00
CR4	−10.70	−9.97	−2.43	−1.84
TS4−1	18.42	18.16	29.55	15.12
IM	−10.92	−10.89	0.26	−10.57
TS4−2	4.39	4.30	15.87	9.55
CP4	−14.26	−13.21	−5.81	−4.68
CH_2O + NDMA + NO^+	40.76	42.12	31.13	0.59

[a] Gas phase: relative energies, enthalpies, and free energies in kilocalories per mole. [b] Aqueous solution: relative energies (RE_w) in water at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level for comparison.

in pH 3.0 to 3.3, the results may also explain the experimental fact that the optimum acidity for the nitrosation of TMA within this pH range.[37,38,41,46]

In aqueous solution, the energy barriers for the non-assisted and water-assisted hydrolysis mechanism in pathway 1 were calculated to be 32.84 and 15.30 kcal/mol, respectively, as shown in Tables 2 and 3. The energy barriers obtained at the CPCM-CCSD(T)/6-311+G(d,p)//PBE1W/CBSB7 level are 32.63 and 13.40 kcal/mol, which are close to the data at the CPCM-CCSD(T)/6-311+G(d,p)//B3LYP/CBSB7 level. This indicates that the water-assisted hydrolysis mechanism is still more favored, which is consistent with the case in the gas phase. Accordingly, a conclusion can be drawn that the water-assisted hydrolysis mechanism is more favored than the non-assisted hydrolysis mechanism and should be the one that is mainly operative. The energy barrier for the transformation from PR3 to CP3 in pathway 2 was calculated to be 25.46 kcal/mol (Table 4). With regard to pathway 3, reaction barriers for the first step reaction (formation of the intermediate IM) and the second step reaction (formation of CP4) were calculated to be 16.96 and 20.12 kcal/mol, respectively. This implies that, under experimental conditions, pathway 1 is more feasible than pathways 2 and 3 for the transformation from the iminium ion to NDMA in aqueous solution. Although the new mechanism described in pathway 3, a stepwise reaction of the iminium ion with N_2O_3, has a relatively higher energy barrier than pathway 1, it is still more favored than pathway 2, a direct reaction of the iminium ion with NO_2^- to form NDMA. Comparing the energy barriers of all steps in the reaction, a conclusion can be drawn that the rate-determining step in aqueous solution is the transformation from the iminium ion to NDMA.

4. Conclusions

The formation mechanisms of NDMA from the nitrosation of TMA were investigated at the CBS-QB3 level of theory. The reaction was proposed to be initiated by the formation of a highly reactive iminium ion, $Me_2N^+{=}CH_2$. Two different pathways, that is, oxidation abstraction and NOH elimination mechanisms, were investigated to elucidate the formation of the iminium ion, and the oxidation abstraction mechanism was found to be more favored than the NOH elimination mechanism. The results not only support the feasibility of the experimentally proposed oxidation abstraction mechanism but also extend this mechanism from the nitrosation of *N*,*N*-dialkyl aromatic amines[63] into the case of tertiary aliphatic amines.

Starting from the iminium ion, three different pathways leading to NDMA were examined. Pathway 1 proposes that the iminium ion undergoes hydrolysis to give a secondary amine DMA, which then can be directly nitrosated to NDMA. Two different hydrolysis mechanisms were examined for this pathway: non-assisted and water-assisted hydrolysis mechanisms. The energy barrier for the water-assisted hydrolysis mechanism was predicted to be almost half the magnitude of the barrier for the non-assisted mechanism, indicating that the former should be predominant. In pathways 2 and 3, the iminium ion reacts with NO_2^- and N_2O_3 to form a neutral and a positively charged intermediate, respectively, which then both collapse to NDMA. Comparing the three pathways in aqueous solution, pathway 1 is the most favored, and pathway 3 has a relatively lower energy barrier than pathway 2. All calculation results indicate that the rate-determining step of the whole reaction in aqueous solution is the transformation from the iminium ion to form NDMA.

On the basis of the theoretical study reported in this article, some experimental results on the *N*-nitrosamines formation from tertiary amine can be explained. This work will be helpful to elucidate the formation mechanisms of *N*-nitrosamines from tertiary amines.

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Supporting Information Available: Absolute energy for each stationary point involved in the formation of NDMA from the nitrosation of trimethylamine. These materials are available free of charge via the Internet at http://pubs.acs.org.

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JP9056219

Exhibit 8

Nitrosative Dealkylation of Some Symmetrical Tertiary Amines

By **Brian G. Gowenlock, Roderick J. Hutchison, (Mrs.) Janet Little,** and **Josef Pfab,*** Department of Chemistry, Heriot-Watt University, Riccarton, Currie, Edinburgh EH14 4AS

The rates of nitrosodealkylation of several symmetrical tertiary amines R_3N including trialkylamines (R = Me, Et, Pr^n, and Bu^n) and substituted trialkylamines (R = $C_6H_5CH_2$, triethanolamine, and nitrilotriacetic acid) have been measured in aqueous acetic acid–acetate buffers. The rate of formation of diethylnitrosamine was found to be first order in nitrous acid, triethylamine, and in the hydrogen ion concentration for pH >3.1. Rates increased with decreasing amine basicity. The rate equation was consistent with rapid, reversible nitrosation by nitrous acid or acetyl nitrite and a rate-determining subsequent elimination.

THE formation of nitrosamines from the reaction of nitrites with tertiary amines was, at one time, discounted. The history of the changing understanding of this reaction system has been reviewed by Hein [1] and by Smith and Loeppky.[2] More recently attention has been directed [3-13] to the public health aspects of the nitrosation of tertiary amines and quaternary ammonium compounds. It has been shown that a wide variety of tertiary amines can react with nitrite in the pH range 3—6.5 and temperature range 37—90° to produce nitrosamines in varying yields. It is claimed [10] that the nitrosation of simple aliphatic tertiary amines in dilute aqueous solution at 100° obeys the kinetic equation (1).

$$\text{Rate} = k[\text{amine}][\text{nitrite}]^3 \qquad (1)$$

This is the only published kinetic study. It seems probable that at these temperatures there will be substantial decomposition of the nitrous acid. A third-power dependence on nitrite concentration is unusual and contrasts with the rate equations obtained for the nitrosation of secondary amines.[14,15] The objectives of the work described here were to define the stoicheiometry and the kinetics of dealkylative nitrosation of some symmetrical tertiary amines in order to judge whether there were some amines which could be subject to this reaction *in vivo*.

EXPERIMENTAL

Materials.—All tertiary amines, with the exception of trimethylamine, were commercially available samples. They were analysed by g.l.c. prior to use. Triethylamine was also checked for removal of any diethylamine by using the procedure of Schweinsberg and Sander.[10] It was noted that there was no difference in the rate of formation of diethylnitrosamine when the purified amine was employed as compared with untreated triethylamine. Trimethylamine was prepared in aqueous solution from reaction of trimethylamine hydrochloride with the stoicheiometric quantity of aqueous sodium hydroxide.

Dialkylnitrosamines were prepared from nitrosation of the secondary amine under standard conditions with the exception of dibenzylnitrosamine where the method of Curtius and Franzen [16] was used. The purity was checked by g.l.c. and t.l.c. prior to spectrophotometric analysis to obtain the molar extinction coefficient.

Qualitative Analysis of the Products of Nitrosation of Triethylamine.—A thoroughly degassed mixture of sodium nitrite and triethylamine dissolved in a sodium acetate–acetic acid buffer solution was heated at 80—90 °C for 2 h and the gaseous products removed by vacuum distillation. Analysis of the products by g.l.c. and i.r. showed the presence of nitrous oxide, acetaldehyde, and diethylnitrosamine.

Determination of the Stoicheiometry of the Nitrosation of Triethylamine.—Nitrosation of triethylamine was carried out at 80 °C for 8 h using reactants as above in a reflux system with an attached trap containing ethanol-free chloroform to absorb volatile products. On termination of the heating, the cooled solution was extracted four times with ethanol-free chloroform including that from the trap and a 10 cm^3 portion of the extract was placed in a standard flask with known amounts of the internal g.l.c. standards carbon tetrachloride and hexadecane. G.l.c. analysis was then carried out using an F and M gas chromatograph (6 ft × 1/4 in column of 10% Carbowax 20M on 60—80 mesh Diatoport W with hydrogen as carrier gas). Temperature conditions were injection port 250, detector 310, column 95 (acetaldehyde and carbon tetrachloride), or 180 °C (diethylnitrosamine and hexadecane).

Kinetic Method.—The reaction mixture was contained in a flask fitted with a thermometer, a reflux condenser with an attached chloroform-containing trap, and a Drechsel bottle head for the withdrawal of samples. The flask, containing buffer solution and amine (100 cm^3), was immersed in the thermostatted water-bath until temperature equilibration was achieved. Sodium nitrite solution (100 ml) at the same temperature was added with shaking to ensure mixing. Samples for analysis were withdrawn at regular intervals and the reaction was quenched by cooling to 0 °C. When the kinetic run was completed, the samples were allowed to reach room temperature and 10 ml of each were taken, saturated with potassium carbonate, and extracted with a small quantity of chloroform, this sample then being made up to 10 ml with chloroform. The u.v. spectrum of each chloroform solution of the dialkylnitrosamine was measured and the concentration of the nitrosamine determined from the optical density at the absorption maximum of nitrosamine. Prior calibrations were made for each nitrosamine studied. Unless stated otherwise all measurements were carried out with a solution of anhydrous sodium acetate (8.5 g) in 60% aqueous acetic acid (100 ml) as buffer providing an initial pH of the mixture of 3.8—3.9.

RESULTS

Reaction Stoicheiometry.—It was shown for triethylamine that 0.9 ± 0.1 mol of acetaldehyde were formed for each mol of diethylnitrosamine formed in good agreement with

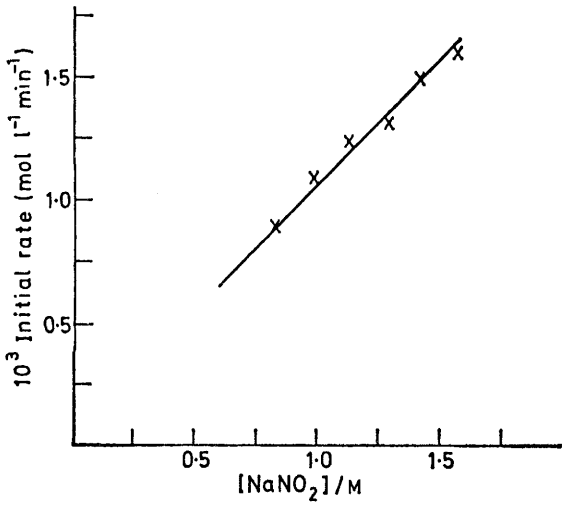

FIGURE 1 Variation of initial rate with nitrite concentration at 74.9 °C and pH 3.8

the stoicheiometry implied by the results of Smith and Loeppky.[2]

Kinetic Studies.—The thermal decomposition of nitrous acid accompanying nitrosodealkylation of the amines precluded the use of integrated rate equations. The less accurate initial rate method had to be employed in which the initial rate is obtained from the slope extrapolated to zero of plots of the nitrosamine concentration against time.

(1) *Triethylamine.* Figures 1—4 give the data for production of diethylnitrosamine. In each set of data one variable (amine concentration, nitrite concentration, pH, and temperature) is altered. The variation of the initial rate of formation of nitrosamine with the stoicheiometric concentration of amine and nitrite shows that equation (2)

$$d[Et_2NNO]/dt = k[\text{triethylamine}][NaNO_2] \quad (2)$$

holds. The actual concentrations of triethylamine and of the nitrous acid present at a given pH were calculated using pK_a values of 10.7 for triethylamine and 3.4 for nitrous acid.

$$d[Et_2NNO]/dt = k'[Et_3N][HNO_2] \quad (3)$$

The rate constants k' obtained in this way are defined by equation (3) and depend on the pH. The variation of k' with pH shows that there is a maximum rate at pH 2.9—3.0. In the pH range 3.1—3.9 this rate coefficient is proportional to the hydrogen concentration as indicated by Figure 5. Thus expression (3) can be expanded for this range to give (4) where k'' is a pH independent rate constant. An

$$d[Et_2NNO]/dt = k''[H^+][Et_3N][HNO_2] \quad (4)$$

investigation of possible anion catalysis was confined to the addition of (a) 0.32M-sodium chloride which gave a reaction mixture of pH 3.70 and no significant increase in the initial rate of nitrosation and (b) 0.32M-potassium thiocyanate which gave a reaction mixture of pH 3.95 and a definite increase in the initial rate of nitrosation accompanied by an orange colouration.

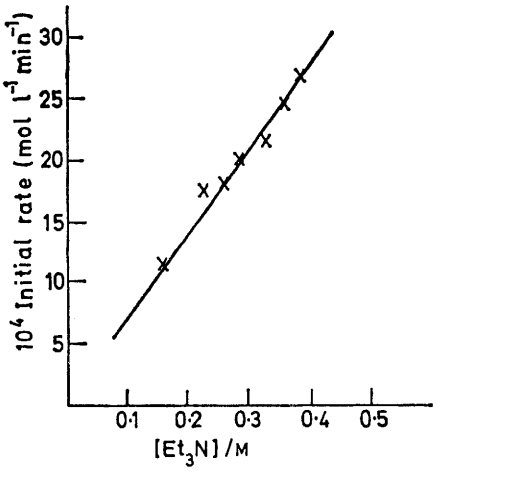

FIGURE 2 Variation of initial rate with triethylamine concentration at 74.9 °C and pH 3.8

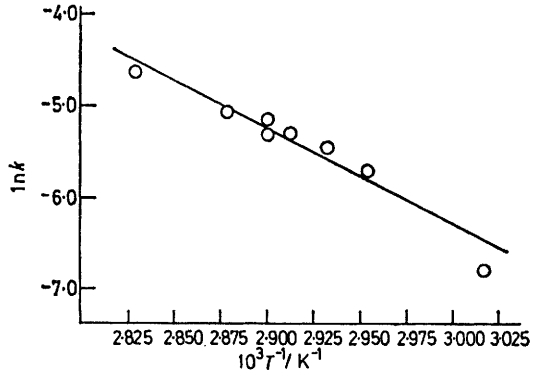

FIGURE 3 Arrhenius plot for the nitrosodealkylation of triethylamine at pH 3.8

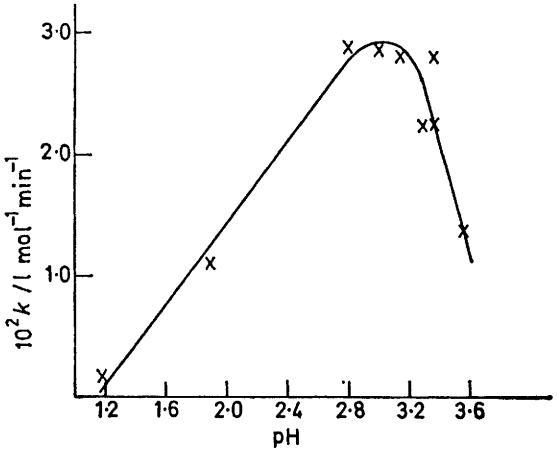

FIGURE 4 Variation of initial rate with pH at 74.8 °C

The activation energy for the nitrosation reaction was obtained from the Arrhenius plot (see Figure 3) giving a value of E of 84.8 kJ mol^{-1} for the temperature range 58—81 °C. The temperature dependence of pK_a was neglected.

Other symmetrical tertiary amines R_3N. It was shown for one further example (R = Bu^n) that the rate of the dealkylation reaction was first order in both the amine and the nitrite. The rates for all other amines were therefore evaluated with the assumption that the empirical rate equation (2) was obeyed. All these studies were conducted at 75° and pH 3.8, the reaction medium consisting of 30% acetic acid buffered by sodium acetate (8.5 g). The concentrations of sodium nitrite required to obtain con-

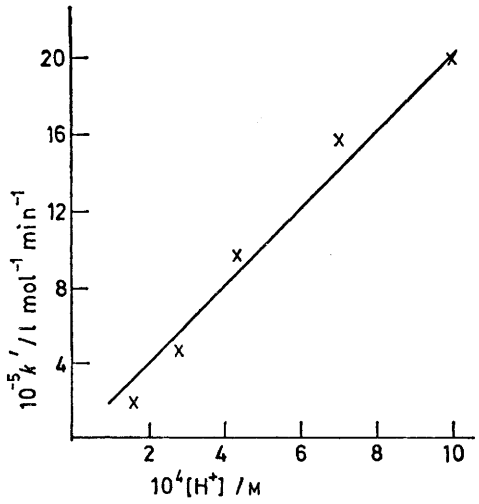

FIGURE 5 Variation of k' with the hydrogen ion concentration at 74.8 °C

veniently measureable rates were between 0.1 and 0.5M, amine concentrations varied from *ca.* 3×10^{-2}M for the most reactive tribenzylamine to 0.15M for the less reactive strongly basic amines. The results obtained are given in the Table and compared with the pK_a values of the amines.[17,18]

Rate constants for the dealkylative nitrosation of tertiary amines R_3N at 75 °C and pH 3.8,

R	$\varepsilon_{max.}$ [a] / l mol^{-1} cm^{-1}	pK_a [b]	10^3k / l mol^{-1} min^{-1}	$10^{-8}k''$ / l^2 mol^{-2} min^{-1}
Et	89.3	10.7	6.4	11
Me	96.4	9.75	8.1	1.6
Pr^n	103	10.7	22	39
HO(CH$_2$)$_2$	83.1	<9.5	25	<2.8
Bu^n	91.1	10.3	30	21
PhCH$_2$	80.4	~8.75	32	~0.63
HO$_2$CCH$_2$	84.0	<9.0	40	<1.4

[a] Extinction coefficients of n,π^*-maxima in chloroform used for the assay of dialkylnitrosamines. [b] Literature values [17] and estimates based on basicity series [18] and extrapolations.

DISCUSSION

The earlier work on the nitrosative dealkylation of tertiary amines was interpreted [1,2,19] in terms of an initial nitrosation of the amine with formation of an *N*-nitrosotrialkylammonium ion and a subsequent *cis*-elimination of nitroxyl to form a tertiary ammonium ion $R_2\overset{+}{N}$=CHR′. The end products, a carbonyl compound and a dialkylnitrosamine, were thought to arise from the hydrolysis of the ammonium ion and the nitrosation of the resulting intermediate secondary amine.[2] The stoicheiometry observed for triethylamine in this work is consistent with such a mechanism. The rate is proportional to the hydrogen ion and stoicheiometric nitrite and triethylamine concentrations in the pH >3 range. The concentration of nitrous acid and of the unprotonated amine are related to these stoicheiometric concentrations by pH-dependent dissociation equilibria, but the rate constants based on the true HNO_2 and amine concentrations are still proportional to the hydrogen ion concentration (Figure 5). Equation (3) can therefore be rewritten for the specified acidity range in the more generalised form (4) using the pH independent rate constant k''.

Equation (4) resembles previously established rate laws for the kinetics of nitrosation or diazotisation of amines.[20] These usually are the result either of slow production of the nitrosating agent or rate-determining nitrosation steps. Both high temperatures and high concentrations of nitrite are required to obtain adequate rates for the nitrosodealkylation reaction. In some of the runs the total initial concentration for the sum of the various nitrosating species exceeds 0.2M. We can therefore rule out the possibility that the rate of this reaction is limited by slow production of the nitrosating agent, within the pH range investigated, and a rate-determining nitrosation may seem more likely. Such a situation is commonly encountered in the nitrosation of secondary amines and the diazotisation of primary amines where the attack of N_2O_3 on the unprotonated amine is rate determining.[20-23] Indeed it appears at first that the pH–rate profile observed in our case (Figure 4) might support such an interpretation. The pH–rate profiles for the nitrosation of simple dialkylamines for instance also exhibit maxima near pH 3.4.[21] These maxima arise because the rate equation contains a second-order dependence in nitrous acid. The linear decrease in the concentration of unprotonated amine is therefore counteracted in these cases by a quadratic increase in the concentration of N_2O_3 as the hydrogen ion concentration increases.

In contrast we have found that the nitrosodealkylation rates exhibit first-order dependence in nitrous acid. The rate maximum at pH 2.9—3.0 cannot therefore be due to rate-determining initial nitrosation by N_2O_3. Consequently we prefer the conclusion that the change in the acidity profile at pH 3 indicates a change in the rate-determining step. A similar situation has been reported previously for the diazotisation of anilines in concentrated aqueous perchloric acid.[24] Although we cannot rule out that the initial nitrosation is rate limiting at pH 3.0, we consider this possibility unlikely at pH 3.7—3.8, where nearly all our measurements with the other amines were performed.

The measurements for different tertiary amines carried out at pH 3.8 (Table) show that there are considerable variations in the rates of nitrosodealkylation. The comparison of the pH-independent rate constants k'' and the pK_a values for the amines indicates that no strict correlation exists between the amine basicities and the rate constants. The n-butyl derivative for example is almost twice as reactive as the ethyl derivative although it is the weaker base. In contrast, the rate constants for the nitrosation of secondary amines by N_2O_3 correlate with amine basicities,[25] as expected if electrophilic nitrosation is rate determining. These results therefore support the conclusion that the initial nitrosation step is not rate limiting and consequently we prefer a mechanism involving relatively rapid and reversible nitrosation followed by slower subsequent steps leading

to the products. With tertiary amines nitrosation results in the formation of nitrosoalkylammonium salts, species that cannot undergo irreversible tautomerisation or loss of a proton as commonly encountered with intermediates in the diazotisation of primary amines or the nitrosation of secondary dialkylamines. Thus the main difference to the nitrosation of primary and secondary amines is the absence of a rapid, irreversible product-forming step. Under such conditions the denitrosation of the nitrosotrialkylammonium ion can not be neglected, and it is necessary to assume that the initial nitrosation is reversible. The frequently acid-catalysed denitrosation of nitrosamines has been established by a considerable body of chemical experience as well as by recent kinetic measurements.[26] Our conclusions are summarised in the Scheme.

$$HNO_2 + HX \underset{}{\overset{K_1}{\rightleftharpoons}} ONX + H_2O$$

$$HX \overset{K_2}{\rightleftharpoons} H^+ + X^-$$

$$R_3N + ONX \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{(1)}{R_3NNO^+} + X^-$$

$$\underset{(1)}{R_3NNO^+} \xrightarrow{k_4} \underset{(2)}{R_2\overset{+}{N}{=}CHR'} + HNO$$

$$R_2\overset{+}{N}{=}CHR' \xrightarrow{k_5} \text{products } (R_2NNO \text{ and } R'CHO)$$

$X^- = OAc^-, NO_2^-, Cl^-, SCN^-$, and H_2O

SCHEME

Application of the steady state principle to the nitrosoammonium intermediate (1) and the assumption that $k_5 \gg k_4$ lead to equation (5) for the rate of product

$$\text{Rate} = K_1 \frac{k_3k_4[H^+][R_3N][HNO_2][HX]}{k_{-3}K_2[HX] + k_4[H^+]} \qquad (5)$$

formation. With the assumption that $k_{-3}K_2[HX] \gg k_4[H^+]$ equation (5) can be simplified to give (6).

$$\text{Rate} = (K_1K_3k_4/K_2)[H^+][R_3N][HNO_2] \qquad (6)$$

Equation (6) reflects the dependence of the measured rates on the concentration of reagents correctly as shown by comparison with (4). We conclude therefore that the Scheme is consistent with the experimental results.

It is noteworthy that no firm conclusions can be drawn with respect to the nature of the active nitrosating species. The observed kinetic order of one with respect to nitrous acid in particular does not eliminate the possibility that the effective reagent is N_2O_3. On the other hand nitrosyl acetate is a likely alternative to N_2O_3 given that the acetic acid concentrations used in this work were high. The negligible effect of added chloride and thiocyanate is explicable if the rates of the nitrosation and denitrosation step are affected equally or if there is little competition of these ions with the excess of acetate and nitrite under the conditions of the reaction. Whilst the formation of nitrosyl chloride may be unlikely in this system, there was clear evidence for the presence of nitrosyl thiocyanate when SCN^- was added. The absence of a marked catalytic effect of this ion may therefore be taken as additional support for the Scheme. This behaviour contrasts with the nitrosation of secondary amines where the addition of thiocyanate leads to considerable increases in the rate of nitrosation when the denitrosation reaction is unimportant.

The activation energy of 85 kJ mol^{-1} observed for the nitrosodealkylation of triethylamine in this work is within the range expected for typical elimination reactions [27] but does not differ much from the values observed for normal *N*-nitrosation reactions.[21-23,28] Typical cyclic *syn*-(E_i) elimination reactions show activation energies which are somewhat higher. Since the substrate (1) is charged, however, significant medium effects will be encountered and an E_i mechanism for the elimination step (1) ⟶ (2) cannot be ruled out.

It is of interest to consider briefly the results of previous studies in the light of our conclusions. Smith and Leoppky contended on the basis of relative cleavage ratios of unsymmetrical amines and product analyses rather than rate measurements that the susceptibility of a tertiary amine to nitrosative cleavage is markedly reduced by base-weakening effects.[2] Wegler and Frank, on the other hand, observed that the ease of cleavage increases in the order cyclic < alkyl < benzyl.[29] Since benzylamines are weaker bases than their alkyl analogues these results seem to be in conflict. It must be remembered, however, that Wegler's observations have to be judged against the pH-dependent rate constants k, in contrast to Smith's which need to be compared with the true pH independent rate constants k''. The fact that there is, however, no correlation of the k'' values with the basicities among the strong amines does indicate that both structural, in particular steric effects, as well as electronic effects in the substrate play an important role in the rate-determining elimination step.

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[8/1107 *Received*, 13*th June*, 1978]

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Exhibit 9

2006 WL 166452
Only the Westlaw citation is currently available.
NOT FOR PUBLICATION
United States District Court, D. New Jersey.

Jeff PLAYER, et al., Plaintiffs,
v.
MOTIVA ENTERPRISES LLC, a successor in interest to Star Enterprises, Defendant.

No. Civ. 02–3216(RBK).
|
Jan. 20, 2006.

Attorneys and Law Firms

Keith A. McKenna, McKenna, Mulcahy & McKenna, Montclair, NJ, for Plaintiffs.

Jeffrey W. Moryan, Connell Foley LLP, Roseland, NJ, for Defendant.

OPINION

KUGLER, United States District Judge:

***1** This matter comes before the Court upon motions by Defendant Motiva Enterprises, LLC, ("Defendant" or "Motiva") for summary judgment of the claims of Plaintiffs Jeff Player, et al. ("Plaintiffs"), and to exclude Plaintiffs' experts Michael Gochfeld, M.D., Ph.D. ("Gochfeld"), R. Brian Ellwood, Ph.D. ("Ellwood"), Bruce M. Gallo ("Gallo"), and Daniel McDonald ("McDonald"). For the reasons set forth below, Defendant's motions will be granted in part and denied in part.

I. Background [1]

This environmental contamination suit is brought by the current and former owners of twenty-seven parcels of residential property located in the Spring Hollow Subdivision in Gloucester Township, New Jersey. [2] Plaintiffs allege that emissions from Defendant's nearby Texaco gasoline service station contaminated their property and the Kirkwood Cohansey Aquifer, the underground water source for their potable wells.

Contamination of the aquifer was first detected on April 5, 2000, when significant concentrations of the gasoline-related compound methyl tertiary butyl ether ("MTBE") was discovered in a drinking fountain at Camden County Community College. New Jersey Consumers Water Company ("Consumers"), the entity responsible for providing water to the college, conducted sampling of some of its wells and discovered significant amounts of gasoline-related compounds in municipal supply well number 8 ("CW–8"). Consumers took the well offline on April 10.

While investigating the contamination, the New Jersey Department of Environmental Protection ("NJDEP") detected a discharge of volatile organic compounds ("VOCs") from Defendant's service station, located at 585 Berlin Cross Keys Road ("Motiva site" or "contamination site"). [3] The NJDEP issued a Field Directive on April 12, 2000, requiring Motiva to investigate the source and extent of the discharge, to implement an interim treatment system, and to submit a remedial action work plan to the NJDEP. Defendant installed an interim recovery system and twenty-five monitoring and recovery wells between April and June 2000.

The NJDEP issued a second directive on May 5, 2000, ordering Defendant to cease gasoline retail operations and provide treatment or an alternate source of water to replace CW–8. Defendant replaced the interim system with a permanent ground water recovery and treatment system in June 2000, and installed forty-one additional monitoring wells from June 2000 to present. As further required by the NJDEP, Defendant regularly sampled potable wells located on approximately forty residential properties in the vicinity of the Motiva site. Defendant detected small amounts of MTBE in thirteen of the residential wells it sampled. [4]

Per the NJDEP directive, Motiva submitted a Remedial Investigation Work Plan/Remedial Investigation report on July 2000 and a Remedial Action Workplan ("RAW") on November 14, 2000. In its RAW, Defendant requested permission to cease sampling of the residential wells, contending that the MTBE detected in those wells could not have come from the Motiva site since the wells are located upgradient [5] or sidegradient from the site, and no emissions were detected in most of the monitoring wells between the Motiva site and the potable wells. [6] Motiva also claimed that recent literature indicated that traces of MTBE in groundwater could likely result from "non-point sources." (March 2001 Directive at 2.)

*2 Plaintiffs' expert, R. Brian Ellwood, Ph.D ("Ellwood"), submitted a response to the RAW on January 17, 2001. In his report, Ellwood notes that as of January 17, 2001, "[c]ontrol of contamination at depth beneath the site, control of offsite contamination, and possibly control of contamination at the northern site boundary, has not been established." (Preliminary Report Sicklerville Road Groundwater Contamination ("Ellwood Report"), McKenna Cert. in Opp. to Def.'s Mot. Summ. J., filed Oct. 12, 2005 ("McKenna Cert."), Ex. F, at 2.) Ellwood also offered possible theories to demonstrate the plausibility of Defendant's responsibility for the MTBE in spite of Motiva's arguments to the contrary.

The NJDEP ultimately rejected Defendant's request to cease sampling of the residential wells in its March 2001 Directive on the basis that "there is insufficient evidence for Equiva to conclude that the MTBE detected in the 13 potable wells in the area did not originate from the Cross Keys Texaco site" and "that regardless of the source of the MTBE in these wells, which is obviously debatable, ongoing sampling of these wells is required *primarily due to their proximity to the site.*" (March 2001 Directive at 2) (emphasis in original).

Also in the March 2001 Directive, the NJDEP approved a Classification Exemption Area ("CEA") for the site that excluded all but 1/10 of an acre of 583 Berlin Cross Keys Road (the Wallace Property). The CEA establishes the boundaries of a ground water plume where VOCs exceed the GWQS. [7]

Through summer 2004, the NJDEP regularly reduced the testing requirements. By August 18, 2003, the NJDEP required only:

> annual sampling of the wells at 4, 7, 11, 13 and 14 Donna Marie Court; 2, 4, 6, and 8 Latham Way; 12 and 20 Spring Hollow Drive, and; 937 and 948 Sicklerville Road. For all the sampling events of the aforementioned potable wells conducted April 2002, the Department notes that all wells continue to exhibit no gasoline related contamination in excess of the Department's Drinking Water Quality Standards.

(NJDEP Directive, Aug. 18, 2003, McKenna Cert., Ex. D.)

The NJDEP approved shut down of the recovery and treatment system on April 30, 2004. (NJDEP Correspondence, Aug. 9, 2004, Mairo Cert., Ex. S., at 2.) Finally, on August 9, 2004, the NJDEP determined that "Defendant's Remedial Action Progress Reports "meet the conditions of the March 21, 2001 Remedial Action Workplan (RAW) approval. Shell Oil Products U.S. (Shell OPUS) is, therefore, in compliance with N.J.A.C. 7:14B–6." (Aug. 9, 2004, NJDEP Correspondence, Mairo Cert., Ex. S., at 1.)

B. The Residential Properties

Plaintiffs own twenty-seven respective residential properties near Defendant's gasoline station. [8] Twenty-six of the twenty-seven properties-all but 583 Berlin Cross Keys Road ("the Wallace property")-contain potable wells located in the Kirkwood Cohansey Aquifer. Because Plaintiffs' properties are north/northeast of the contamination site, (Undisputed Facts ¶ 38), they are considered upgradient or sidegradient of the contamination site, depending on whether CW–8 is pumping. [9]

*3 Consistent with the requirements of the NJDEP directives, Defendant tested the Plaintiffs' residential wells for six gasoline-related compounds: benzene, toluene, ethylbenzene, xylenes, MTBE, and TBA. No testing detected any gasoline-related compound on eighteen of the properties. [10] Detection of compounds on the remaining eight properties was as follows:

- A single detection of 0.79 ppb toluene and ten detections of MTBE (highest at 15.5 ppb) at 4 Latham Way,
- Three detections of MTBE (highest at 0.76 ppb) at 14 Donna Marie Court,
- Three detections of MTBE (highest at 1.4 ppb) at 6 Latham Way,
- A single detection of 1.4 ppb toluene at 850 Sicklerville Road,

- A single detection of 0.4 ppb MTBE at 4 Donna Marie Court,
- A single detection of 0.3 ppb MTBE at 12 Donna Marie Court,
- A single detection of 1.2 ppb MTBE at 8 Latham Way, and
- A single detection of 0.3 ppb MTBE at 20 Spring Hollow Road.

The GWQS for toluene is 1,000 ppb and the GWQS for MTBE is 70 ppb. No gasoline-related compound was detected on any Plaintiff's property after April 2001.

According to the Certification of Julian Davies, a Project Manager for EnviroTrac, Ltd., an environmental consulting firm retained by Defendant to remediate the Motiva site, the NJDEP never restricted the consumption of water from Plaintiffs' potable wells, and never required Defendant to treat the water, provide Plaintiffs with an alternate source of water, or collect soil samples from the residential properties. [11] (Julian Davies Cert., Mairo Cert., Ex. R, at 2.)

Since the fact of the contamination became known, several Plaintiffs have sold their property. Maria and John Wallace sold 583 Berlin Cross Keys Road for $350,000.00 in September 2001, Plaintiffs Thomas and Tina Stankiewicz sold 9 Spring Hollow Drive in July 2002 for $143,000.00, Barbara Tanner sold 17 Spring Hollow Drive for $134,000.000 in February 2002, Daniel and Maria Rodriguez sold 18 Spring Hollow Drive for $138,000.00 in July 2003, David Lodi sold 5 Donna Marie Court for $104,000.00 in September 2001, 13 Donna Marie Court was sold for $109,900.00 in July 2000, and 19 Spring Hollow Drive was sold for $133,900.00 in May 2001.

Defendant filed motions for summary judgment and to exclude experts on June 24, 2005, after requesting and receiving permission from this Court to extend by one week the date for the filing of dispositive and *in limine* motions. Briefs in opposition were due July 22, 2005, however, Plaintiffs instead filed an untimely request for an extension on August 2, 2005, and a second request on September 6, 2005, moving the deadline to September 30, 2005. On October 5, 2005, Plaintiffs filed another untimely request for an extension, and ultimately did not submit a complete Opposition until October 14, 2005. Nevertheless, because a district court should not grant a motion for summary judgment without examining the merits, *Stackhouse v. Mazurkiewicz*, 951 F.2d 29, 30 (3d Cir.1991) (citing *Anchorage Assoc. v. Virgin Islands Bd. of Tax Rev.*, 922 F.2d 168 (3d Cir.1990)), this Court will exercise its discretion to consider Plaintiffs' Opposition, even though it is untimely. Local Civ. R. 7.1(d)(5).

II. Standard

*4 Summary judgment is appropriate where the Court is satisfied that "there is no genuine issue as to any material fact and that the moving party is entitled to a judgment as a matter of law." Fed.R.Civ.P. 56(c); *Celotex Corp. v. Catrett*, 477 U.S. 317, 330, 106 S.Ct. 2548, 91 L.Ed.2d 265 (1986). A genuine issue of material fact exists only if "the evidence is such that a reasonable jury could find for the nonmoving party." *Anderson v. Liberty Lobby, Inc.*, 477 U.S. 242, 248, 106 S.Ct. 2505, 91 L.Ed.2d 202 (1986).

The burden of establishing the nonexistence of a "genuine issue" is on the party moving for summary judgment. *Celotex*, 477 U.S. at 330. The moving party may satisfy this burden by either (1) submitting affirmative evidence that negates an essential element of the nonmoving party's claim; or (2) demonstrating to the Court that the nonmoving party's evidence is insufficient to establish an essential element of the nonmoving party's case. *Id.* at 331.

Once the moving party satisfies this initial burden, the nonmoving party "must set forth specific facts showing that there is a genuine issue for trial." Fed.R.Civ.P. 56(e). To do so, the nonmoving party must "do more than simply show that there is some metaphysical doubt as to material facts." *Matsushita Elec. Indus. Co. v. Zenith Radio Corp.*, 475 U.S. 574, 586, 106 S.Ct. 1348, 89 L.Ed.2d 538 (1986). Rather, to survive summary judgment, the nonmoving party must "make a showing sufficient to establish the existence of [every] element essential to that party's case, and on which that party will bear the burden of proof at trial." *Serbin*, 96 F.3d at 69 n. 2 (quoting *Celotex*, 477 U.S. at 322); *Heffron v. Adamar of N.J., Inc.*, 270 F.Supp.2d 562, 568–69 (D.N.J.2003). "If the non-movant's evidence on any essential element of the claims asserted is merely 'colorable' or is 'not significantly probative,' the court must enter summary judgment in favor of the moving party."

Heffron, 270 F.Supp.2d at 69 (citing *Anderson,* 477 U.S. at 249–50).

III. Motion to Exclude Expert Daniel McDonald

Defendant moves to exclude the testimony of Plaintiffs' expert Daniel McDonald ("McDonald") on the grounds that he is unqualified and his report is unreliable. [12] Admissibility of expert testimony is governed by Federal Rule of Evidence 702 and the United States Supreme Court's decision in *Daubert v. Merrell Dow Pharmaceuticals, Inc.,* 509 U.S. 579, 113 S.Ct. 2786, 125 L.Ed.2d 469 (1993). [13] In the Third Circuit, the admissibility of expert testimony is contingent on the "qualifications" of the expert and the "reliability" of his methodology. *In re Paoli R.R. Yard PCB Litig.,* 35 F.3d 717 (3d Cir.1994) (interpreting *Daubert*); *see also Oddi v. Ford Motor Co.,* 234 F.3d 136, 145 (3d Cir.2000).

A. *In Limine* Hearing

In certain instances, courts are obligated to provide *in limine* hearings before applying *Daubert* to exclude expert testimony. *Padillas v. Stork–Gamco, Inc.,* 186 F.3d 412 (3d Cir.1999). A hearing is required, for example, where the court excludes an expert's conclusions on the grounds that they are "insufficiently explained and the reasons and foundations for them inadequately and perhaps confusingly explicated." *Id.* In other words, where a report is "conclusory and did not adequately explain the basis for [the expert's] opinion or the methodology employed in reaching his conclusions," the "plaintiff needs an 'opportunity to be heard' on the critical issues of scientific reliability and validity." *Oddi,* 234 F.3d 136, 152 (3d Cir.2000) (holding that the district court did not err "in granting summary judgment here without an *in limine* hearing") (quoting *Padillas,* 186 F.3d at 417). Where the evidentiary record is substantial, however, or the court has before it the information necessary to determine that the expert lacks "good grounds" for his conclusions, an *in limine* hearing may be unnecessary. *Id.* at 153.

***5** The evidence before this Court clearly establishes the process by which McDonald "arrived at his conclusions," *Oddi,* 234 F.3d at 152, and McDonald's report and deposition details the methodology underlying his determinations. As discussed below, this Court will exclude McDonald's testimony on the grounds that his analysis and methodology are baseless and inconclusive, not because his report is insufficiently explained. Additionally, Defendant's motion for summary judgment alerted Plaintiffs to the *Daubert* challenge, yet Plaintiffs neither requested a hearing nor offered any affidavit or evidence in support of McDonald. Accordingly, an *in limine* hearing is unnecessary.

B. Qualifications

The Third Circuit instructs courts to "liberally" evaluate an expert's qualifications. *Oddi v. Ford Motor Co.,* 234 F.3d 136, 145 (3d Cir.2000). In particular, the Circuit has "eschewed overly rigorous requirements of expertise and [has] been satisfied with more generalized qualifications." *In re Paoli,* 35 F.3d at 741 (citing *Hammond v. International Harvester Co.,* 691 F.2d 646, 652–53 (3d Cir.1982) and *Knight v. Otis Elevator Co.,* 596 F.2d 84, 87–88 (3d Cir.1979)). This liberal treatment extends to the expert's substantive qualifications as well as his formal qualifications. *Id.*

Nevertheless, the Third Circuit has "also set a floor with respect to an expert witness's qualifications." *Elcock v. Kmart Corp.,* 233 F.3d 734, 742 (3d Cir.2000). To demonstrate when an expert would not be qualified under Rule 702, the *Elcock* Court offered the pre-*Daubert* case, *Aloe Coal Co. v. Clark Equip. Co.,* 816 F.2d 110 (3d Cir.1987), which held a tractor salesperson unqualified to testify as an expert about the cause of a tractor fire. *Elcock,* 233 F.3d at 742 (citing *Aloe Coal,* 816 F.2d 110).

In *Elcock* itself, the Court determined with "misgivings" that the district court had not abused its discretion by concluding that a psychologist with experience in obtaining employment for disabled individuals was qualified to testify to the possibility for vocational rehabilitation of the injured plaintiff. However, the Court acknowledged that it also would have upheld a decision to exclude the expert since "he seems most qualified to testify on a micro-level regarding the ability of a disabled individual to return to a specific job; he does not appear particularly qualified to testify on the macro-level regarding the number of jobs in the national or local economy that the disabled individual is able to perform." [14] *Elcock,* 233 F.3d at 744. Taken together, *Elcock* and *Aloe Coal* indicate that where a proposed expert's area of experience is

adjacent to, but not actually encompassing, the subject matter of his testimony, he may be deemed unqualified.

McDonald has worked as a licensed appraiser in New Jersey for approximately twenty-two years. Defendant argues that McDonald is nevertheless unqualified to testify to the diminution in value of Plaintiffs' properties because McDonald has no experience in appraising contaminated property. Defendant notes that McDonald has never appraised property allegedly contaminated by emissions from a gasoline station and has never acted as an expert in a situation involving contamination of the groundwater or allegations of a leaking underground storage tank. (Daniel McDonald Dep. ("McDonald Dep."), Mairo Cert. in Supp. Def.'s Mot. to Exclude Plaintiffs' Expert Daniel McDonald, Ex. C, at 23–24.) Defendant also points out that McDonald did not entirely understand the Ellwood and Gallo reports upon which he relied, including the charts indicating the presence and degree of contaminating agents on the property. (McDonald Dep. at 55–56.)

***6** This case lies squarely between *Aloe Coal* and *Elcock*. Although McDonald is an experienced appraiser, no evidence indicates that he has any experience appraising contaminated properties or is qualified to value the effects of stigma on property values. Just as a psychologist experienced in assisting individuals to find work may be unqualified to testify about the general availability of jobs in the economy, an individual able to appraise an uncontaminated property may have no grounds for appreciating the devaluation of the same property under unique conditions of contamination or stigma. Because nothing in McDonald's experience indicates knowledge or expertise in issues of contamination, he is unqualified to testify to the loss of value to Plaintiffs' properties arising from the alleged contamination.

C. Reliability

Because expert testimony has the potential to bear considerable weight with a jury, the district court functions as a gatekeeper responsible for assuring "that the scientific methodology upon which the expert opinion is founded is reliable" and that "the expert's conclusion is based on good grounds." *In re Paoli*, 35 F.3d at 732–33. To ascertain "reliability," the court must examine a number of factors, both those established in *Daubert* and those previously enumerated by the Third Circuit in *United States v. Downing*, 753 F.2d 1224 (3d Cir.1985). *Oddi*, 234 F.3d 145 (citing *Paoli II*, 35 F.3d at 742). In particular, the court must consider:

> (1) whether a method consists of a testable hypothesis; (2) whether the method has been subjected to peer review; (3) the known or potential rate of error; (4) the existence and maintenance of standards controlling the technique's operation; (5) whether the method is generally accepted; (6) the relationship of the technique to methods which have been established to be reliable; (7) the qualifications of the expert witness testifying based on the methodology; and (8) the non-judicial uses to which the method has been put.

Paoli II, 35 F.3d at 742 n. 8; *see also* *Elcock*, 233 F.3d at 746 (noting that "each factor need not be applied in every case"). The party wishing to introduce the testimony bears the burden of establishing "by a preponderance of the evidence that their opinions are reliable." *Paoli*, 35 F.3d at 744.

Of course, an expert's opinion need not be "perfect," and judges may not substitute their opinions for those of an expert. *Paoli*, 35 F.3d at 744; *see also* *Crowley v. Chait*, 322 F.Supp.2d 530, 536 (D.N.J.2004). However, courts also need not admit mere conclusions or "opinion evidence that is connected to existing data only by the *ipse dixit* of the expert. A court may conclude that there is simply too great an analytical gap between the data and the opinion proffered." *Magistrini v. One Hour Martinizing Dry Cleaning*, 180 F.Supp.2d 584, 608 (D.N.J.2002) (quoting *General Elec. Co. v. Joiner*, 522 U.S. 136, 145–46, 118 S.Ct. 512, 139 L.Ed.2d 508 (1997)).

***7** Mere assumptions, without causal evidence or methodological analysis may be inadmissible. *In re TMI Litig.*, 193 F.3d 613, 667–68 (3d Cir.1999). Conclusions based only on the expert's experience, *Oddi*, 234 F.3d at 140–41, and testimony founded on methods that are not

generally accepted or lack testable hypotheses may also fail to surmount the *Daubert* standard, *Elcock,* 233 F.3d at 746. Furthermore, conclusions based on analogies that are too dissimilar to the subject of the testimony may also merit exclusion. *General Elec.,* 522 U.S. at 144 (rejecting expert testimony that plaintiff's cancer was due to exposure to PCBs when the testimony was based on animal studies of infant mice that had developed cancer after exposure to PCBs).

In response to Defendant's motion to exclude McDonald's testimony, Plaintiffs argue that "Mr. McDonald's opinions are based upon credible facts, NJDEP records, the reports of Plaintiffs' liability experts and individual appraisal reports prepared for each residential property." (Pl.s' Opp. Def.'s Mot. Summ. J. ("Opp."), filed Oct. 12, 2005, at 30.) However, McDonald testified in his deposition that he relied only on the Gallo and Ellwood Reports, and he specifically testifies that he did *not* "review any correspondence from the NJDEP related to this site." (McDonald Dep. at 15.) [15]

In spite of Plaintiffs' arguments to the contrary, this Court cannot avoid the conclusion that McDonald's methodology is entirely unreliable. In his report, McDonald determines that the value of Plaintiffs' properties with no evidence of contamination should be discounted 35% percent and property with onsite contamination should be discounted by 66%. (McDonald Report ("McDonald Report"), Mairo Cert. in Support of Def.'s Mot. Exclude Pl.s' Expert Daniel McDonald, Ex. B., at 31, 33.) McDonald reached the 35% and 66% figures without discussing, or even recognizing, the extent to which the property was actually contaminated. As demonstrated by his ignorance of the "ND"/Not Detected signifier in the Gallo and Ellwood Reports, McDonald did not know how to read the charts denoting the levels of contamination. (McDonald Dep. at 56.) Nor had McDonald ever conducted any physical inspection of or visit to the properties prior to writing the report. [16] (McDonald Dep. at 15–16.)

Furthermore, to quantify the stigma attached to Plaintiffs' properties, McDonald relies upon a highly misleading analogy with a site of profoundly contaminated residential properties in Dover Township. (McDonald Report at 27.) Specifically, McDonald compares Plaintiffs' properties with "an area of Dover Township that had ground water contamination from Union Carbide and ... Ciba Geigy that resulted in what was commonly known as a cancer cluster among children," meaning "an inordinate number of children with cancer." (McDonald Dep. at 158–59.) McDonald selected the Dover site not because of its comparability, but because McDonald "didn't know of any other cases that, where the data was as readily available." (McDonald Dep. at 159.)

***8** Employing the Dover analogy, McDonald determined that the property in the Dover site is in the final stages of recovery and continues to suffer a stigma loss of 13%. Because McDonald considered Plaintiffs' properties in the early stages of recovery, McDonald determined that they must bear a stigma discount of at least two or three times that of the Dover site, resulting in a discount of 35%. [17] However, the severity of the contamination and resulting illness among Dover residents undercuts any grounds for comparison with Plaintiffs' properties where there were few detections of contaminants and no reported physiological effects.

The methodology employed to reach the 66% figure is equally unreliable. To assess the value of properties with some evidence of contamination, McDonald sent an email to thirteen financial lenders to determine whether they would "lend on a property that has known contamination, or the stigma of contamination, to the ground water." (McDonald Report at 32.) Of the thirteen lenders, six replied. One of those refused to comment, and one said that it would loan given certain circumstances. The other four lenders stated that they would not lend on a property that is contaminated, but the content of their brief responses suggested that they understood the email hypothetical to denote property that was actually contaminated and out of compliance with state requirements. [18]

From the results of the email test, McDonald concludes that there would be no buyers other than those who could pay cash. [19] McDonald then assessed the discount in value given cash-only buyers, extrapolating from this a discount of 66%. (McDonald Report at 33.) However, the reliability of the 66% figure is entirely invalidated by the overemphasis placed on the four responses to the email hypothetical, the misleading implication in the email hypothetical, suggesting a much greater contamination of the property than actually present, and the unclear calculations and assumptions underlying McDonald's arrival at 66%.

Ultimately, McDonald's report does not fulfil any of the reliability factors. His method is untestable and arbitrary, without a generally accepted, established, or peer reviewed methodology, and his evaluation was conducted without any

real standards. Because McDonald is unqualified and his evaluation is unreliable, Defendant's motion *in limine* to exclude his testimony will be granted.

IV. Plaintiffs' Claims

A. Negligence and Gross Negligence

To surmount a motion for summary judgment of a negligence claim, Plaintiffs must provide evidence such that a reasonable jury could find "breach of a duty of care and actual damages sustained as a proximate cause of the breach." *Muise v. GPU, Inc.*, 371 N.J.Super. 13, 35, 851 A.2d 799 (App.Div.2004) (citing *Weinberg v. Dinger*, 106 N.J. 469, 484, 524 A.2d 366 (1987)); *Nappe v. Anschelewitz, Barr, Ansell & Bonello*, 97 N.J. 37, 45, 477 A.2d 1224 (1984) ("[T]he plaintiff must show a breach of duty and resulting damage to prevail in a negligence action."). Motiva argues that Plaintiffs have failed to establish damages and causation and requests summary judgment of Plaintiffs' gross negligence claim on the same basis. [20]

*9 The absence of an injury will preclude a negligence claim, even where a clear breach of duty is present. *Rocci v. MacDonald–Cartier*, 323 N.J.Super. 18, 24–25, 731 A.2d 1205 (App.Div.1999) (affirming summary judgment for insufficient evidence of damages in defamation case and noting that "a plaintiff must present proof of a material question of fact as to both liability and damages") (citing *Norwood Easthill Assoc. v. Norwood Easthill Watch*, 222 N.J.Super. 378, 384, 536 A.2d 1317 (App.Div.1988) (affirming summary judgment of malicious interference claim on basis that "plaintiff has suffered no injury or damage")). At the summary judgment stage, Plaintiffs must provide actual evidence of injury and cannot simply rely upon "unsubstantiated allegations." *Trap Rock Indus., Inc. v. Local 825*, 982 F.2d 884, 890 (3d Cir.1992) (reversing district court's denial of summary judgment). Just as "a residential customer not in residence during a power loss, or a commercial customer whose store was closed, might have no damages except the inconvenience of resetting clocks," *Muise*, 371 N.J.Super. at 49, 851 A.2d 799, the release of contaminants into the groundwater aquifer does not itself generate damages, unless Plaintiffs can show that they suffered harm.

Plaintiffs concede that they "have not presented and will not present claims for the present manifested bodily injury." (Undisputed Facts ¶ 67.) However, they argue that they have adequately established damages for medical monitoring and property damage. They do not address their claim for emotional distress. [21]

1. Medical Monitoring

Damages for medical monitoring are appropriate where a plaintiff exhibits no physical injury, but nevertheless requires medical testing as a proximate result of a defendant's negligent conduct. *Ayers v. Jackson Twp.*, 106 N.J. 557, 600, 525 A.2d 287 (1987). The risk of injury need not be quantified to merit medical surveillance damages; however, the plaintiff must establish that the risk of serious disease is "significant." *Id.* at 599–600, 525 A.2d 287; *Campo v. Tama*, 133 N.J. 123, 131, 627 A.2d 135 (1993) (awarding medical monitoring damages to a plaintiff with a "fifty-to seventy-five-percent chance of suffering a recurrence of cancer" due to the delay resulting from defendant doctor's malpractice). In the case of toxic exposure, "medical-surveillance damages may be awarded only if a plaintiff reasonably shows that medical surveillance is required because the exposure caused a distinctive increased risk of future injury." *Theer v. Philip Carey Co.*, 133 N.J. 610, 627, 628 A.2d 724 (1993). Such damages are "not available for plaintiffs who have not experienced direct and hence discrete exposure to a toxic substance and who have not suffered an injury or condition resulting from that exposure." *Id.* at 628, 628 A.2d 724.

Low level contamination, "that is, contamination below the minimum level set by DEP for water remediation," typically is insufficient to establish injurious toxic exposure. *Muralo Co., Inc. v. Employers Ins. of Wausau*, 334 N.J.Super. 282, 290–291, 759 A.2d 348 (App.Div.2000) ("[S]ince it is clear that no untreated groundwater is ever entirely pure, we are satisfied that DEP standards are the most reliable guide for determining whether contamination causing damage ... has occurred."). Here, contaminants have been detected in only eight of Plaintiffs' wells, and no detection has been even close to the GWQS. The NJDEP never restricted Plaintiffs' use of water from their potable wells, nor required Defendant to treat Plaintiffs' wells or to provide Plaintiffs with an alternate water source.

*10 Plaintiffs rely on the testimony of Dr. Michael Gochfeld, Ph.D. ("Gochfeld"), to establish the significant health risks

and necessity of medical surveillance following from the alleged contamination of Plaintiffs' property. However, nothing in Gochfeld's report concludes that the individual Plaintiffs themselves require medical monitoring under the circumstances. Rather, Gochfeld's report creates a medical monitoring program for a hypothetical target population without taking into consideration the actual exposure of any plaintiff.[22] (Gochfeld Dep. at 26–29.) Gochfeld prepared his report under the assumption that "there were known or actual or potential exposure to a variety of constituents of gasoline." (Gochfeld Dep. at 12.) He states in deposition that he had "no specific factual knowledge of the actual exposures in this case," and he confirms that he has never examined the individual Plaintiffs. (Gochfeld Dep. at 10, 29.)

Gochfeld himself notes that "[w]hether a person exposed to MTBE requires medical monitoring depends in large measure on the level of exposure and the time over which it occurred" and notes that "clearly people that are exposed to MTBE casually would not require one." (Gochfeld Dep. at 24.) Furthermore, Gochfeld stated that he "probably would not" recommend medical monitoring for the minor and often single detections of MTBE on Plaintiffs' properties.[23] (Gochfeld Dep. at 46–50.) Consequently, Gochfeld's report does not establish that Plaintiffs require medical monitoring.

Plaintiffs also appear to argue that their wells may have been more contaminated prior to the initiation of Defendant's testing in July 2000. (Opp. at 20.) However, Plaintiffs provide no evidence suggesting that such exposure actually occurred or that any exposure prior to July 2000 was more than minimal. Plaintiffs also argue for the first time in their Opposition that they may have ingested water from contaminated sources besides the potable wells on their property. (Opp. at 20.) However, Plaintiffs offer no evidence that any Plaintiff actually consumed water from CW–8. Without any evidence supporting their theories, Plaintiffs cannot establish a claim for medical monitoring sufficient to survive summary judgment.

Because Plaintiffs have provided no evidence of a "distinctive increased risk of future injury" from the exposure, Plaintiffs are not entitled to damages for medical monitoring.

2. Property Damage

Defendant requests summary judgment of Plaintiffs' claims of property damage on the grounds that the contamination caused no actual damage to Plaintiffs' properties.[24] Instead of claiming that their property was physically harmed, Plaintiffs contend that the news of the contamination stigmatized their property, reducing its value in the minds of potential buyers.

In support of their claim for stigma damages, Plaintiffs offer the expert testimony of Daniel McDonald. However, as discussed previously, McDonald's testimony must be excluded as unreliable. Plaintiffs also argue that the testimony of individual Plaintiffs establishes a stigma discount to their property:

> ***11** Plaintiff Marie Wallace has submitted sworn Interrogatory statements documenting a $150,000.00 loss on the sale of her property. See Exhibit 0 to McKenna Certification. Other Plaintiffs have similarly provided certified answers to Interrogatories and Deposition testimony as to the loss in value through sales transactions, which occurred from the discharge. See Exhibit N–R to the McKenna Certification.

(Opp. at 20–21.)

This evidence fails to establish an injury. Exhibits N–R consist of contracts for sale and unexecuted contracts for sale of three of Plaintiffs' properties, including the Wallace property, leaving it to the Court's imagination to ascertain how these contracts demonstrate a loss in value. Wallace's testimony also fails to establish a stigma injury to the property.

Specifically, Wallace claims that she received a verbal offer for her asking price of $500,000.00 from a man named "Amin," whose last name she cannot recall. (Marie Wallace Dep., McKenna Cert., Ex. 0, M.) Wallace claims that he reneged from the agreement after she told him about the release, however, the alleged offeror never gave Wallace the offer in writing and she has no evidence of the offer or "Amin's" motive for withdrawing, aside from her own testimony. Consequently, even construing this evidence in the light most favorable to Plaintiffs, no reasonable jury could find that Plaintiffs' properties were stigmatized on the basis of this evidence alone.

3. Emotional Distress

Defendant also moves for summary judgment of Plaintiffs' claim for emotional distress. Plaintiffs do not respond to this argument in their Opposition, and Defendant is entitled to summary judgment of Plaintiffs' emotional distress claim for Plaintiffs' failure to present evidence of significant distress or physical injury.

A claim for emotional distress cannot succeed absent evidence of physical injury or "severe and substantial" emotional distress, even where a person has a reasonable concern of an enhanced risk of future disease. *Ironbound Health Rights Advisory Com'n v. Diamond Shamrock Chem. Co.*, 243 N.J.Super. 170, 174–75, 578 A.2d 1248 (App.Div.1990) (noting that "[i]n the absence of physical injury, damages are allowed where the resultant emotional distress is severe and substantial" and listing cases). Without some physical injury, mere exposure to toxic chemicals does not give rise to a claim for emotional distress damages. *Id.* (holding plaintiffs unable to sustain emotional distress claim for exposure to chemicals manufactured at plant near their residences); *see also* *Mauro v. Raymark Indus., Inc.*, 116 N.J. 126, 137, 561 A.2d 257 (1989); *Troum v. Newark Beth Israel Med. Ctr.*, 338 N.J.Super. 1, 17, 768 A.2d 177 (App.Div.2001). Because Plaintiffs provided no evidence of significant emotional distress or physical injury, Defendant's motion for summary judgment will be granted.

B. Trespass

Defendant moves for summary judgment of Plaintiffs' claim for trespass. Plaintiffs argue that Defendant's "intentional refusal" to remove the contamination from their property and failure to install remediation equipment amounts to an intentional trespass.[25] (Opp. at 25.)

***12** The Restatement (Second) of Torts defines intentional trespass as:

> One who intentionally and without a consensual or other privilege
>
> (a) enters land in possession of another or any part thereof or causes a thing or third person so to do, or
>
> (b) remains thereon, or
>
> (c) permits to remain thereon a thing which the actor or his predecessor in legal interest brought thereon in the manner stated in §§ 160 and 161, is liable as a trespasser to the other irrespective of whether harm is thereby caused to any of his legally protected interests.

Rest. (2d) Torts § 158.

As Defendant argues, New Jersey has moved away from "such common law claims as trespass and nuisance" in environmental pollution cases. *Mayor and Council of Borough of Rockaway v. Klockner & Klockner,* 811 F.Supp. 1039, 1053 (D.N.J.1993); *Kenney v. Scientific, Inc.*, 204 N.J.Super. 228, 256, 497 A.2d 1310 (1985) ("There is no need for us ... to torture old remedies to fit factual patterns not contemplated when those remedies were fashioned."). Regardless of the continuing viability of trespass claims in the environmental context, however, Plaintiffs have failed to come forward with any evidence supporting their claim and cannot survive summary judgment.

Plaintiffs note that they are "not arguing that Defendants intentionally caused the contamination of their property," but rather are claiming that "defendants have repeatedly refused to perform the horizontal and vertical delineation of the soil and groundwater contamination in the area of the residential properties." (Opp. at 25.) However, no evidence suggests that such measures were necessary to remove contaminants from Plaintiffs' properties. Rather, the record indicates that Defendant consistently complied with NJDEP requirements, including the installation and maintenance of a groundwater recovery system to rehabilitate the aquifer, and the NJDEP never required Defendant to install any sort of remediation equipment on any of the residences. Given that there has been no detection of a gasoline-related contaminant in any Plaintiff's potable well since April 2001, the argument that Defendant permitted contamination to remain on Plaintiffs' properties lacks any viable evidentiary foundation. Defendant's motion for summary judgment of Plaintiffs' trespass claim will be granted.

C. Strict Liability

Plaintiffs originally claimed a cause of action for strict liability under the theory that the handling, storage, or use of gasoline constitutes an abnormally dangerous activity. However, Plaintiffs voluntarily dismissed this claim in their Opposition. (Pl.'s Opp. at 3.) Accordingly, the Court will not address the merits of Plaintiffs' strict liability claim.

D. Environmental Statutes

1. New Jersey Environmental Rights Act

Plaintiffs allege a right to recover under the New Jersey Environmental Rights Act ("ERA"), N.J.S.A. 2A:35A–1 *et seq.* Defendant requests summary judgment on the grounds that Plaintiffs have not satisfied the ERA's notice provision, N.J.S.A. 2A:35A–11, and that an ERA claim is not actionable where the NJDEP has acted to institute and oversee remediation of the contamination.

***13** Section 4(a) of the ERA, permits "any person" to "maintain an action in a court of competent jurisdiction against any other person to enforce, or to restrain the violation of, any statute, regulation or ordinance which is designed to prevent or minimize pollution, impairment or destruction of the environment." N.J.S.A. 2A:35A–4(a). Although the ERA itself does not create substantive rights, it confers standing on private persons to enforce other environmental statutes, including the New Jersey Spill Compensation and Control Act ("Spill Act"). *Rockaway,* 811 F.Supp. at 1054; *Allied Corp. v. Frola,* 701 F.Supp. 1084, 1091 (D.N.J.1988).

The NJDEP is "entrusted initially with the right to determine the primary course of action to be taken." *Howell Township v. Waste Disposal, Inc.,* 207 N.J.Super. 80, 95, 504 A.2d 19 (App.Div.1986) ("In order to be effective, [the NJDEP] must normally be free to determine what solution will best resolve a problem on a state or regional basis given its expertise and ability to view those problems and solutions broadly."). Consequently, the right of private parties to sue under the EPA is "an alternative to inaction by the government which retains primary prosecutorial responsibility." *Superior Air Prod. Co. v. NL Indus., Inc.,* 216 N.J.Super. 46, 58, 522 A.2d 1025 (App.Div.1987); *Rockaway,* 811 F.Supp. at 1054 ("[T]he primary goal of the ERA is to limit lawsuits by private litigants to those instances where the government has not acted.").

A private ERA suit may be permitted even in the absence of complete government inaction if the NJDEP has "failed in its mission ... failed or neglected to act in the best interest of the citizenry or has arbitrarily, capriciously or unreasonably acted." *Howell,* 207 N.J.Super. at 96, 504 A.2d 19; *Morris County Transfer Station, Inc. v. Frank's Sanitation Serv., Inc.,* 260 N.J.Super. 570, 578, 617 A.2d 291 (App.Div.1992) (permitting private ERA action where the NJDEP would not address violation for three years and had taken no enforcement actions against contaminating defendant who continued operating its illegal facility two months after receiving a violation notice). Where NJDEP "action subsequently proves sufficient to protect the environment," however, NJDEP "action under the Spill Act is preemptive of private rights under ERA." *Superior Air Prod.,* 216 N.J.Super. at 61, 522 A.2d 1025. The permissibility of private action must be evaluated on a case-by-case basis. *Id.*

Here the record indicates consistent and pervasive NJDEP oversight of the remediation process, requiring Defendant to regularly test Plaintiffs' wells and institute interim and permanent groundwater recovery systems. Plaintiffs have not claimed that the NJDEP failed to act or acted unreasonably, and there are no grounds for finding NJDEP inaction sufficient to permit a private ERA suit. Furthermore, as discussed below, Plaintiffs failed to give the NJDEP the requisite notice of their private suit. Accordingly, Defendant's motion for summary judgment of Plaintiffs' ERA claim will be granted.

2. Notice

***14** Before a private party may commence an action under the ERA, the party must "at least 30 days prior to the commencement thereof, direct a written notice of such intention by certified mail, to the Attorney General, the Department of Environmental Protection, the governing body of the municipality in which the alleged conduct has, or is likely to occur, and to the intended defendant." N.J.S.A. 2A:35A–11. The notice provision is intended to give the government an adequate opportunity to intervene in the litigation and to allow the NJDEP:

> to exercise value judgments in individual cases, e.g., whether it will join in that litigation or enforcement proceeding, whether other actions it may have taken already with respect to the particular problem or offender would render the litigation subject to collateral estoppel or res judicata principles, whether its expertise would assist the court, whether broad State interests would be sacrificed unduly to

> regional or personal interests by the instigators of that litigation, etc.

Howell, 504 A.2d at 95; *Morris County,* 260 N.J.Super. at 578, 617 A.2d 291 (quoting *Howell* for same).

Because Plaintiffs did not provide the required thirty day notice to the NJDEP or the Attorney General, they are barred from further pursuing their claim under the ERA. Plaintiffs argue that Defendant is judicially estopped from claiming lack of notice for failure to raise this issue at an earlier stage in the case. Plaintiffs analogize the ERA requirement to that of an affidavit of merit, required in certain cases to avoid "unmeritorious and frivolous malpractice lawsuits at an early stage of litigation." *Knorr v. Smeal,* 178 N.J. 169, 197–98, 836 A.2d 794 (2003) (holding judicially estopped defendant's request for summary judgment for plaintiff's failure to file affidavit of merit) (citing *Palanque v. Lambert–Woolley,* 168 N.J. 398, 404, 774 A.2d 501, 505 (2001)); *Ferreira v. Rancocas Orthopedic Assoc.,* 178 N.J. 144, 836 A.2d 779, (2003) (same).

Defendant argues that the ERA notice requirement is more analogous to the notice of intent in the Resource Conservation and Recovery Act (RCRA), which the Supreme Court held to be a jurisdictional prerequisite to suit in *Hallstrom v. Tillamook County,* 493 U.S. 20, 31, 110 S.Ct. 304, 107 L.Ed.2d 237 (1989) ("[C]ompliance with the 60–day notice provision is a mandatory, not optional, condition precedent for suit."); *Public Interest Research Group of N.J., Inc. v. Windall,* 51 F.3d 1179, 1189 (3d Cir.1995) (holding notice provision jurisdictional in context of Clean Water Act ("CWA")); *Hawksbill Sea Turtle v. Federal Emergency Mgmt. Agency,* 126 F.3d 461, 471 (3d Cir.1997) (holding notice provision jurisdictional in context of Endangered Species Act ("ESA")).

However, the language of the notice requirement in RCRA is not entirely analogous to that of the ERA. RCRA states, under the heading of "Actions prohibited" that "No action may be commenced ... prior to 60 days after the plaintiff has given notice of the violation to" the Administrator, the state and the alleged violator. 42 U.S.C.A. § 6972. The ERA lacks the "no action may be commenced" language of the RCRA, CWA, and ESA, and states only that notice must be sent "at least 30 days prior to the commencement" of suit. Consequently, the argument that the plain language of the statute creates a jurisdictional bar is not as strong in the context of the ERA.

***15** Nevertheless, because the purpose of the notice provision is to provide the Attorney General and NJDEP with notice of the suit and opportunity to intervene, *Howell,* 504 A.2d at 95, and not merely to protect defendants, as in the case of the affidavit of merit, Defendant is not judicially estopped from raising Plaintiffs' lack of compliance with the notice provision and is entitled to summary judgment of Plaintiffs' ERA claim.

E. Spill Act Claim

In their complaint, Plaintiffs assert a private right of action under the Spill Act, N.J.S.A. 58:10–23.11 *et seq.*[26] As amended in 1991, the Spill Act authorizes a private cause of action for individuals to recover costs for environmental damage to their property. *Housing Auth. of City of New Brunswick v. Suydam Inv., L.L.C.,* 177 N.J. 2, 18, 826 A.2d 673 (2003). Actions under the Spill Act are limited to clean up and removal costs, *Bahrle v. Exxon Corp.,* 145 N.J. 144, 155, 678 A.2d 225 (1996), defined as:

> all direct costs associated with a discharge, and those indirect costs that may be imposed by the department pursuant to section 1 of P.L.2002, c. 37 associated with a discharge, incurred by the State or its political subdivisions or their agents or any person with written approval from the department in the: (1) removal or attempted removal of hazardous substances, or (2) taking of reasonable measures to prevent or mitigate damage to the public health, safety, or welfare, including, but not limited to, public and private property.
>
> N.J.S.A. 58:10–23.11b(d). The Act does not authorize "damages arising from emotional distress, enhanced risk of disease, loss of enjoyment of property, and other economic and financial harm." *Bahrle,* 145 N.J. at 155, 678 A.2d 225.

Plaintiffs maintain that the investigation conducted by Ellwood was a reimbursable clean up and removal cost under the Spill Act. As Plaintiffs suggest, because "a discharge cannot be addressed until the contaminants are defined and the extent of the discharge determined," certain forms of investigative costs are implicitly included in the Act. *Metex Corp. v. Federal Ins. Co.,* 290 N.J.Super. 95, 115, 675 A.2d 220 (App.Div.1996).

However, for a private party to obtain reimbursement under the Act, the party must have obtained "written approval from the department," for example, in a memorandum of agreement, prior to incurring the cost. N.J.S.A. 58:10–23.11b(d); *Id.* Such approval permits the NJDEP to "review and approve or disapprove its investigation to date, its proposed remedial action, and its report of the implementation of its action." *Id.; see also* *Interfaith Cmty Org. v. Honeywell Intern., Inc.*, 263 F.Supp.2d 796, 867 (D.N.J.2003) (concluding "that such costs were approved by and/or incurred at the direction of NJDEP and thus are recoverable under the Spill Act."). Because Plaintiffs have not obtained NJDEP approval for any cost incurred, including the Ellwood report, Defendant is entitled to summary judgment of Plaintiffs' Spill Act Claim.

***16** The accompanying Order shall enter today.

Elcock, 233 F.3d at 741.

All Citations

Not Reported in F.Supp.2d, 2006 WL 166452

Footnotes

1 The following facts are taken from Defendant's statement of undisputed material facts, filed June 24, 2005, ("Undisputed Facts") and Plaintiffs' counterstatement of undisputed facts, filed Oct. 14, 2005, ("Counterstatement Facts"). Plaintiffs did not provide a separate statement of undisputed facts.
Although Plaintiffs dispute the majority of Defendant's statements of fact, Plaintiffs' counterstatements typically provide additional facts without setting forth any conflicting evidence. Where no actual disputes are presented, Defendant's statements will be treated as undisputed. *See e.g.,* *Tofano v. Reidel,* 61 F.Supp.2d 289, 292 n. 1 (D.N.J.1999) (citing Fed.R.Civ.P. 56(e)) ("This court will ... not consider assertions without evidential support as creating genuine issues of disputed fact."); *Talbot v. United States,* 2005 WL 2917463, *2 (D.N.J.2005) (noting that where the nonmoving party does not submit facts in opposition, "it is entirely appropriate for this court to treat all facts properly supported by the movant to be uncontroverted") (quoting *Allebach v. Sherrer,* No. 04–287, 2005 U.S. Dist. LEXIS 15626, at *5 (D.N.J.2005)).
More generally, Plaintiffs' brief suffers from numerous typographical errors and a dearth of citations to page numbers in the record. This "alone warrants exclusion of the evidence." *See* *Orr v. Bank of America, NT & SA,* 285 F.3d 764, 774–75 (9th Cir.2002) (holding that party's failure to cite page and line numbers when referencing the deposition merits exclusion of evidence); *Huey v. UPS, Inc.,* 165 F.3d 1084, 1085 (7th Cir.1999) ("[J]udges need not paw over the files without assistance from the parties."); *Nissho–Iwai Am. Corp. v. Kline,* 845 F.2d 1300, 1307 (5th Cir.1988) (parties must designate specific facts and their location in the record).

2 Among the original litigants to the suit were also former plaintiffs Michael and Susan Kammerhoff and Norma Simmons. The Kammerhoff plaintiffs were voluntarily dismissed, and plaintiff Norma Simmons died on August 26, 2000.

3 VOCs generally associated with gasoline discharge include MTBE, benzene, toluene, ethylbenzene, xylene (collectively "BTEX"), and tertiary butyl alcohol ("TBA"). The NJDEP has issued a Ground Water Quality Standard ("GWQS") for each of these VOCs, also known as "gasoline-related compounds." MTBE, for example, has a GWQS of 70 parts per billion ("ppb").

4 Although Motiva detected MTBE in thirteen residential wells, not all of these wells are owned by Plaintiffs to this litigation. Of the twenty-seven parcels of property at issue in this suit, only eight of the properties contain wells that ever tested positive for any gasoline-related compound.

5 The direction of water's flow in an aquifer is described as "downgradient," and the direction against the current is "upgradient."

6 In particular, testing revealed emissions in monitoring wells 6–Shallow ("MW–6S") and 7–Deep ("MW–7D"), which lie between the Motiva site and the residential properties. However, the majority of upgradient monitoring wells did not test positive for gasoline-related contaminants. (NJDEP Directive, March 21, 2001 ("March 2001 Directive"), Mairo Cert. in Supp. Def.'s Mot. Summ. J., filed June 24, 2005 ("Mairo Cert."), Ex. O, at 4.)

7 Plaintiffs dispute Defendant's characterization of the CEA, (Counterstatement Facts ¶ 31), on the basis that Defendant proposed the CEA prior to conducting an actual delineation of the plume and that "the Plaintiffs' residential wells could only had [sic] been included in the CEA, if Defendant intended to supply a permanent public water supply to Plaintiffs' properties." While Plaintiffs' contention with the CEA is not entirely clear, Plaintiffs have not provided any evidence indicating that the NJDEP improperly approved the CEA or that the CEA was an inaccurate representation of the boundaries of contaminants in excess of the GWQS.

8 Plaintiffs' properties are: 850 Sicklerville Road; 565, 569, 581, and 583 Berlin–Cross Keys Road; 6, 9, 10, 12, 13, 14, 1, 16, 17, 18, 20 Spring Hollow; 2, 4, 6, and 8 Latham Way; 3, 4, 5, 7, 12, 14, and 15 Donna Marie Court.

9 CW–8 is located approximately 1,000 feet downgradient of the contamination site. (March 2001 Directive at 2.) While active, CW–8 pumps approximately 500 gallons per minute and causes the groundwater to flow southwest. (Ellwood Report at 2.) When CW–8 is not pumping, the groundwater flow is more westerly. (Ellwood Report at 2.)

10 Plaintiff disputes these facts on the basis that:

> The Defendant has no data for any portable [sic] water supply of the Plaintiffs prior to July 2000. The Defendant never performed any delineation of the groundwater plume in the areas of the residential properties despite having actual knowledge of such contamination in MW–6, MW–7 and MW–12. See Gallo Certification and Exhibits C, D and E.

(Counterstatement Facts ¶¶ 46–48.) However, because Defendant makes no averment of the presence or absence of contamination prior to July 2000, Defendant's statements are not actually in dispute. Plaintiffs provide no fact indicating an inaccuracy in Defendant's statements regarding the testing of Plaintiffs' wells. Consequently, there is no actual dispute regarding the presence or amount of *detected* gasoline-related compounds.

11 Plaintiffs dispute these statements by citing to Exhibit F of the McKenna certification; however, Exhibit F is the Ellwood report and therefore is not indicative of the NJDEP requirements. Plaintiffs nowhere cite to a statement by the NJDEP requiring Defendant to treat their water or provide them with an alternate water source, and therefore this fact is undisputed.

12 Because this Court will grant Defendant's motion for summary judgment, it will not reach the merits of Defendant's motions to exclude experts Gochfeld, Ellwood, and Gallo.

13 After *Daubert,* Rule 702 was amended to encompass the *Daubert* analysis:

> If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion or otherwise, if (1) the testimony is based upon sufficient facts or data, (2) the testimony is the product of reliable principles and methods, and (3) the witness has applied the principles and methods reliably to the facts of the case.

Fed.R.Evid. 702. While *Daubert* itself addressed only the admissibility of scientific evidence, the Court has since noted that courts' gatekeeping obligations extend to all expert testimony. *Kumho Tire Co. v. Carmichael,* 526 U.S. 137, 151, 119 S.Ct. 1167, 143 L.Ed.2d 238 (1999).

14 The Court noted that it had "misgivings" about the expert's qualifications in spite of:

> (1) [the expert's] general training in "assessing" individuals, which he received while earning his Ph.D. in psychology; (2) his experience, twenty years previous, helping drug addicts reenter the workforce; (3) his experience primarily in the last two years dealing with the Virgin Islands Division of Workers' Compensation, which he had advised regarding the ability of approximately fifty to sixty-five disabled employees to return to their previous jobs; (4) his past experience as an expert witness making lost earning capacity assessments;

(5) his attendance at two seminars regarding vocational rehabilitation, and his stated familiarity with the literature in the area; (6) his membership in two vocational rehabilitation organizations, both of which place no restrictions on membership; and (7) the fact that when [the expert] was in school, a degree in vocational rehabilitation therapy was not available, but that he received similar training nonetheless.

15 Plaintiffs also argue that "Defendant does not attack the methodology, standard or factual basis for the opinions," (Opp. at 31), however, it is quite clear from Defendant's motion that the reliability of McDonald's methodology is hotly disputed.

16 McDonald also appeared unaware of the fact that Plaintiffs' properties are served by potable wells, even though the potable wells contain the evidence of contamination.

Q: Do you know whether or not the plaintiffs' properties have potable wells?

A: It's my understanding that they are hooked to a public water system.

Q: If each of the properties did in fact have a potable well, would that be a factor that you were consider relevant in your analysis?

Mr. McKenna: You may want to review the documents that you referenced in your report to assist you in this area. Just separate the Ellwood and Gallo reports. I'm going to go to the men's room.

(Whereupon, a recess is taken.)

Mr. Mairo: I am going to object that Mr. McKenna was basically coaching the witness.

Back on the record.

A: Your question about whether or not each of these houses were, was, had their own private well on site-

Q: Uh-huh?

A: -it's my understanding that each house is served by wells within and around the neighborhood and that Consumer, Consumers Water Company owns those wells and supplies that water to the homes.

(McDonald Dep. at 36.)

17 McDonald reaches the 35% devaluation figure with the following methodology:

The subject properties are in the early stages of monitoring, and clean up of the ground water contamination. The properties from Dover Twp. are beyond the clean up stage and into the final stage of recovery, yet they still show a 13% loss in value as compared to similar properties outside of the contaminated area. The subject area is in stage D of recovery, which is the beginning of the remediation process. Based on the acceptance of the Detrimental Condition Model as a viable process for valuing Detrimental Conditions to Real Estate, by the appraisal community and the Subcommittee on Housing and Community Opportunity of the House Committee on Financial Services, it would be logical to assume that the discount to the properties which are the subject of this report, would be 2 to 3 times that of properties in the final stage of recovery. In this case a discount of 35% would be considered reasonable.

(McDonald Report at 31.)

18 Interbay Funding, for example, qualified their statement that they would not lend by noting, "The property would have to be completely cleaned up. They would have to file all necessary documents to the state of NJ and we would require something from the state telling us the property is cleaned up." (McDonald Report at 32.) From this, McDonald concluded that Interbay Funding would not lend on properties such as Plaintiffs', without considering that none of Plaintiffs' properties were contaminated in excess of state standards.

19 In evaluating this data, McDonald states:

The lenders that did respond have overwhelmingly stated that they would not approve the loan at all, or they would require substantial conditions to the loan. In the case of the subject properties, it can be assumed that a purchaser with private financing or cash would be the only potential buyer of houses in this area.

(McDonald Report at 32.)

20 Because the Court now finds that there is no evidence of any actual injury arising from Defendant's negligence, this Court will not address Defendant's causation argument.

21 Plaintiff argues that Defendant's motion for summary judgment of its negligence claim should be denied on the basis of the doctrine of *res ipsa loquitur*. However, *res ipsa loquitur* acts only to "permit[ ] an inference of defendant's negligence" (i.e., that defendant acted in an unreasonable manner) under particular

circumstances. *Jerista v. Murray,* 185 N.J. 175, 192, 883 A.2d 350 (2005). The doctrine does not establish either causation or the presence of damages. *See e.g., Bahrle v. Exxon Corp.,* 279 N.J.Super. 5, 35, 652 A.2d 178 (App.Div.1995) (holding *res ipsa* doctrine inapplicable where "there was a factual dispute as to whether the contamination was a result of plaintiffs' own voluntary acts or neglect"). Accordingly, because Defendant is contesting only causation and damages, the *res ipsa* doctrine does not apply.

22 Gochfeld testifies in his deposition that he created his report without any specific information about the Plaintiffs:

> Q: So, for example, in determining the percentage of the target population that was in high exposure category, that wasn't based on the ground water, your review of the ground water tables that were attached to Mr. Gallo's report?
> A: It was not.
> Q: That was based purely on just an assumption of yours?
> A: It was an assumption based on experience with previous programs or programs that are currently underway in our communities.
> Q: Having no specific factual knowledge of the actual exposures in this case?
> A: That's correct, these are hypotheticals.

(Gochfeld Dep. at 28–29.)

23 Gochfeld also states that he would not even recommend medical monitoring for the one property with by far the highest detection of MTBE (13.8 ppb at 4 Latham Way) "on this data alone" because "[i]t is possible that a person living there would only be drinking bottled water, would not be in the house very much." (Gochfeld Dep. at 50.)

24 Defendant argues further that New Jersey law does not permit Plaintiffs to recover for stigma damages in the absence of some physical harm to their property. Because Plaintiffs have provided no evidence of any stigma to their property, the Court will not reach Defendant's alternative argument.

25 It is unclear whether Plaintiffs allege negligent trespass since they discuss only the Restatement (Second) of Torts § 158, Intentional Trespass, in their Opposition. Unlike intentional trespass, negligent or reckless trespass requires evidence of "harm to the land, to the possessor, or to a thing or a third person." Rest. Torts 2d § 165; *see also Burke v. Briggs,* 239 N.J.Super. 269, 271, 571 A.2d 296 (App.Div.1990) (citing Rest.2d Torts § 158 with approval for another premise); *Karpiak v. Russo,* 450 Pa.Super. 471, 481, 676 A.2d 270 (Pa.Super.1996) (affirming dismissal of trespass claim for entry of dust onto property since the "evidence failed to establish that the dust caused appellants harm"). As discussed previously, Plaintiffs have not provided any evidence of injury to their persons or property. Consequently, to the extent that Plaintiffs are claiming negligent trespass, Defendant is entitled to summary judgment.

26 It is unclear whether Plaintiffs also raise a claim for cleanup and removal costs from the Spill Compensation Fund under N.J.S.A. 58:10–23.11g(a). (Opp. at 12–13.) However, the appropriate procedure to obtain compensation under the Fund is by filing a claim with the administrator of the Fund, "not later than one year after the date of discovery of damage. The administrator shall prescribe appropriate forms and procedures for such claims." N.J.S.A. 58:10–23.11k. In the event "a party, including a potentially responsible party ... contests the amount or validity of" a claim for reimbursement from the Spill Fund, "the dispute is referred to an arbitrator whose decision may be appealed to the Appellate Division," and the arbitrator's decision will be final unless it was "arbitrary, capricious, or unreasonable." *Lacey Municipal Util. Auth. v. New Jersey Dept. of Envir. Prot., Envir. Claims Admin.,* 369 N.J.Super. 261, 273, 848 A.2d 843 (App.Div.2004). Accordingly, this is an improper forum for a Spill Compensation Fund claim.

End of Document

Exhibit 10

Guidance for Industry

Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Comments and suggestions regarding this draft document should be submitted within 60 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions regarding this draft document contact David Jacobson-Kram at 301-796-0175.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

December 2008
Pharmacology and Toxicology

Guidance for Industry

Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

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Contains Nonbinding Recommendations

Draft — Not for Implementation

Guidance for Industry[1]
Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

This guidance is intended to inform pharmaceutical manufacturers of the Food and Drug Administration's (FDA's) current thinking regarding genotoxic and carcinogenic impurities in drug substances and drug products, including biologic products that are regulated by the Center for Drug Evaluation and Research (CDER). This guidance provides recommendations on how to evaluate the safety of these impurities during clinical development (investigational new drug applications (INDs)) and for marketing applications (new drug applications (NDAs), biologics license applications (BLAs), and abbreviated new drug applications (ANDAs)). This guidance provides recommended exposure thresholds on the clinical exposure to genotoxic or carcinogenic impurities. Also provided are additional testing and exposure threshold recommendations for situations where there are known or theoretical safety concerns based on available data, structural alerts, and/or assessment of the synthetic pathway.

This guidance is intended as an adjunct to the ICH guidances for industry *Q3A(R2) Impurities in New Drug Substances*, *Q3B(R2) Impurities in New Drug Products*, and *Q3C(R3) Impurities: Residual Solvents* that deal with the topic of impurities in a more general fashion.[2] This guidance provides specific recommendations regarding the safety qualification of impurities with known or suspected genotoxic or carcinogenic potential while the ICH guidances provide only general direction. This guidance addresses synthetic impurities and degradants in drug substances, but does not otherwise address the genotoxicity or carcinogenicity of actual drug substances or intended drug product ingredients. This guidance also applies to known starting materials or anticipated reaction products.

[1] This guidance has been prepared by the Office of New Drugs in the Center for Drug Evaluation and Research (CDER) at the Food and Drug Administration.

[2] See http://www.fda.gov/cder/guidance/index.htm. The FDA has incorporated revision 3 (R3) of ICH Q3C into the guidance for industry *Q3C — Tables and List*, which is posted on the CDER guidance Web site.

This guidance describes a variety of ways to characterize and reduce the potential lifetime cancer risk associated with patient exposure to genotoxic and carcinogenic impurities both during clinical development and after approval. These approaches include:

- Changing the synthetic and/or purification routes to minimize the formation and/or maximize the removal of the relevant impurity.

- Allowing a maximum daily exposure target of 1.5 µg per day for the relevant impurity as a general target for marketed products, though higher levels may be acceptable during clinical development. Certain impurities with structural alerts suggesting particularly high genotoxic and carcinogenic potential would not be appropriate for this general threshold approach and would need to be evaluated on a case-by-case basis.

- Further characterizing the genotoxic and carcinogenic risk via mechanism of action or weight-of-evidence approaches, or through additional studies to better support appropriate impurity specifications.

This guidance also applies to drug products approved before the issuance of this guidance, but only in the presence of a specific safety signal that suggests the potential for an increased carcinogenic risk associated with the presence of an impurity or degradant, or with regard to a supplemental application for a previously approved drug product that proposes a significant change in the drug product's approved labeling that suggests the potential for an increased carcinogenic risk associated with the presence of an impurity or degradant (e.g., new indication, new dosage regimen, longer duration of use). Applicants also should take these recommendations into consideration when preparing supplemental manufacturing submissions to NDAs, BLAs, and ANDAs, such as submissions proposing new formulations or new synthetic routes. Although this guidance applies to impurities present in biologic products regulated by CDER, it is noted that, in most cases, the genotoxicity assays conducted for small molecule pharmaceuticals are not applicable to biopharmaceuticals. Likewise, the standard assessment of the genotoxic potential of impurities in biopharmaceuticals may not be appropriate in many cases since they may include residual host cell proteins and nucleic material, fermentation components, and bacterial and viral components and do not include organic chemicals typically found in small molecule manufacturing.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

Compounds that have been demonstrated to induce genetic mutations, chromosomal breaks, and/or chromosomal rearrangements are considered genotoxic and have the potential to cause

cancer in humans. Exposures to even low levels of these impurities may be of significant concern. Therefore, the identification limits provided in ICH Q3A(R2) and ICH Q3B(R2) may not be acceptable for genotoxic or carcinogenic impurities. For instance, under some scenarios the limits in these ICH guidances would allow a genotoxic or carcinogenic impurity to be present in a drug product at a level resulting in exposures up to 3,000 µg per day without needing identification. Although genotoxic and carcinogenic properties can be acceptable for some active pharmaceutical ingredients (APIs) depending on clinical circumstances (e.g., cancer chemotherapies), impurities in drug substances and drug products generally do not have beneficial effects and may impose a risk without associated benefit. Therefore, manufacturers should strive to achieve the lowest levels of genotoxic or carcinogenic impurities that are technically feasible and/or levels that convey no significant cancer risk.

Currently available guidances that address issues related to impurities and residual solvents include ICH Q3A(R2), ICH Q3B(R2), and ICH Q3C(R3). In addition, the European Medicines Agency's (EMEA) Committee for Medicinal Products for Human Use (CHMP) published a guideline regarding limits of genotoxic impurities.[3] These documents are discussed below to provide a background to this guidance, but the inclusion of the EMEA guideline in this background discussion should not be interpreted as an FDA endorsement of that document.

A. ICH Guidances for Industry Relating to Drug Impurities and Residual Solvents

ICH Q3A(R2) and ICH Q3B(R2) address the issue of impurities in drug substances and drug products, respectively. ICH Q3A(R2) addresses the identification and qualification of impurities in drug substances approved after the issuance of the guidance, and ICH Q3B(R2) addresses only those impurities in drug products approved after the issuance of the guidance that are classified as degradation products of the drug substance or reaction products of the drug substance with an excipient and/or immediate container closure system. These guidances define an impurity as any component of the drug substance or drug product other than the chemical entity that makes up the drug substance or an excipient in the drug product. Depending on the quantity of drug substance or drug product to which a patient is exposed, these guidances recommend thresholds for the identification, reporting, and qualification of impurities. *Qualification*, as defined by the two guidances, is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity (or degradation product) or a given impurity (or degradation) profile at the level(s) specified.[4] Higher or lower thresholds for qualification can be considered appropriate based on scientific rationale and level of concern.[5]

These guidances recommend when, after consideration of factors such as the patient population and duration of use, qualification studies of an impurity are appropriate. Part of the battery of tests used to qualify an impurity could include assays to determine whether the impurity is

[3] Guideline on the Limits of Genotoxic Impurities (EMEA guideline), June 2006 (http://www.emea.europa.eu).

[4] See the Glossary sections in ICH Q3A(R2) and ICH Q3B(R2).

[5] See ICH Q3A(R2), section VII, and ICH Q3B(R2), section VI.

genotoxic.[6] These guidances also recommend that, when considered appropriate, assays to assess genotoxic potential include the "minimum screen" of in vitro assays: a gene mutation assay and a chromosomal aberration assay.[7] ICH Q3A(R2) indicates that "such studies can be conducted on the new drug substance containing the impurities to be controlled, although studies using isolated impurities can sometimes be appropriate."[8] A similar recommendation is included in ICH Q3B(R2).

It should be noted, however, that allowing genotoxicity assessment of the impurity as it is present with the drug substance, rather than in isolation, renders the genotoxicity assessments much less sensitive. For example, the potent mutagens that are typically used as positive controls in the bacterial mutation assay, such as 9-aminoanthracene and methyl methanesulfonate, when present with a noncytotoxic drug substance at the minimal level for qualification, would not be detected by these genotoxicity assays because the maximum concentration of the impurity at the limit concentration of the drug substance would not be sufficient to produce a genotoxic response in the assays. If the drug substance is cytotoxic, this approach of assessing the impurity as it is present with the drug substance would be even more insensitive, since the drug's toxicity would further limit the level at which the impurity could be tested.

Although the ICH guidances provide some recommendations on the types of tests that should be conducted, the guidances do not provide specific recommendations on how to proceed if one or both of the genetic toxicology tests are positive; they simply state that additional testing, removal of the impurity, or lowering the level of the impurity should be considered.

ICH Q3C(R3) recommends acceptable concentration limits or permissible daily exposures for various classes of solvents, which are one type of impurity. The guidance does not, however, include a recommendation on limiting exposure based upon concerns for genotoxic potential. The guidance recommends only that mathematical models be used for setting exposure limits in cases where reliable carcinogenicity data are available.

The ICH guidances on impurities and residual solvents do not apply to drug substances or drug products used during the clinical research stages of development.

B. EMEA Proposed Guideline on Limits of Genotoxic Impurities

In June 2006, the EMEA's CHMP published a guideline on the limits of genotoxic impurities in support of a marketing application.[9] A subsequent CHMP safety working party published a

[6] See ICH Q3A(R2), section VII and Attachment 3, and ICH Q3B(R2), section VI and Attachment 3.

[7] Ibid.

[8] See ICH Q3A(R2), section VII.

[9] EMEA guideline (http://www.emea.europa.eu)

question and answers document to provide clarification on the 2006 guideline.[10] This guideline recommends dichotomizing genotoxic impurities into those for which there is "sufficient (experimental) evidence for a threshold-related mechanism" and those "without sufficient (experimental) evidence for a threshold-related mechanism." The genotoxic impurities with sufficient evidence for a threshold-related mechanism would be addressed using methods outlined in ICH Q3C(R3) for Class 2 solvents. This approach calculates a "permitted daily exposure," which is derived using the "no observed effect level" or, alternatively, the "lowest observed effect level" from the most relevant animal study and incorporating a variety of uncertainty factors. Examples of genotoxic compounds that might fall into this category include compounds that induce aneuploidy by interfering with the mitotic spindle, compounds that interfere with the activity of topoisomerase, and/or compounds that inhibit DNA synthesis.

For genotoxic impurities without sufficient evidence for a threshold-related mechanism, the guideline proposes a policy of controlling levels to "as low as reasonably practicable" (called the *ALARP principle*). The ALARP approach specifies that every effort should be made to prevent the formation of such impurities during drug substance synthesis and, if that is not possible, technical effort should be made post-synthesis to reduce impurities (e.g., purification steps). Compounds that fall into this category are those that interact with DNA either directly or indirectly, such as alkylating agents, intercalating agents, or agents that can generate free radicals. Since any exposure to these agents can convey some level of carcinogenic risk, and since complete elimination of genotoxic impurities from drug substances is often unachievable, the presence of a concerning impurity requires the implementation of a concept of an acceptable risk level. Methods for the derivation of acceptable risk levels are discussed in ICH Q3C(R3), Appendix 3, in reference to Class 1 carcinogenic solvents.

Although the approach described above is acceptable, in most instances mechanistic data sufficient to allow for an assessment of whether there is a threshold mechanism are lacking. Furthermore, it is relatively uncommon for there to be sufficient data to allow for a quantitative risk assessment. The EMEA guideline recognizes these limitations and, therefore, proposes the use of a "threshold of toxicological concern" (TTC) for genotoxic impurities. The TTC refers to a threshold exposure level to compounds that does not pose a significant risk for carcinogenicity or other toxic effects. The EMEA guideline recommends a TTC of 1.5 µg per day for all but a highly potent subset of compounds. This threshold corresponds to an incremental 10^{-5} lifetime risk of cancer, a risk level that the EMEA considers justified because of the benefits derived from pharmaceuticals. The guideline indicates that a TTC value higher than 1.5 µg per day may be acceptable based on a weight-of-evidence approach to the profile of genotoxicity results, in situations where the anticipated human exposure will be short-term, for the treatment of life-threatening conditions, when life expectancy is less than 5 years, or where the impurity is a known substance and human exposure will be much greater from other sources. The derivation of the TTC is discussed in more detail in section IV.B.1.

The approach taken in the EMEA guideline for setting an exposure limit for genotoxic or carcinogenic impurities in drug products in support of a marketing application is reasonable. However, issues regarding the presence of genotoxic or carcinogenic impurities often occur

[10] Question & Answers on the CHMP Guideline on the Limits of Genotoxic Impurities, June 2008 (http://www.emea.europa.eu)

during the clinical development stages. Therefore, this guidance provides recommendations for acceptable exposure thresholds during clinical development as well as for marketing applications.

III. RECOMMENDED APPROACHES FOR INITIAL ASSESSMENT OF GENOTOXIC POTENTIAL OF IMPURITIES

If adequate data characterizing genotoxic and carcinogenic potential are not already available, impurities identified in drug substances or drug products at levels exceeding the stated qualification thresholds in the relevant ICH guidances should be assessed for genotoxic potential in an initial minimal screen. Assays conducted with the impurity in isolation are recommended. However, studies with the drug substance containing, or spiked with, the impurity can be considered in cases where it can be demonstrated that synthesizing sufficient amounts of the impurity is infeasible.

As mentioned, the ICH guidances on impurities do not apply to drug substances or drug products for use in clinical trials. However, in cases where the presence of an impurity with genotoxic or carcinogenic potential is identified or where such an impurity may be expected based on the synthetic pathway, steps should be taken during the clinical development stage to address safety concerns associated with these impurities.

If an impurity that is present at levels below the ICH qualification thresholds is identified, the impurity should be evaluated for genotoxicity and carcinogenicity based on structural activity relationship (SAR) assessments (i.e., whether there is a *structural alert*). This evaluation can be conducted via a review of the available literature or through a computational toxicology assessment; commonly used software includes MDL-QSAR, MC4PC, and Derek for Windows. The conduct of an in vitro mutation assay (i.e., bacterial reverse mutation assay) generally would be an acceptable initial screen for impurities with an identified alert, since positive signals in computational toxicology programs are often derived from the results of bacterial mutation assays and mutagenic carcinogens are considered to operate through nonthreshold-related mechanisms. An assessment in a mammalian cell assay may be needed for impurities with specific structural groups, such as carbamates, that are not well characterized in bacterial assays, or for compounds that are toxic to *E. coli* and *Salmonella*, such as antibiotics.

If the initial evaluation of the genotoxic potential of an impurity is negative, no further genotoxicity studies are recommended and the impurity should be considered to be adequately qualified regarding its genotoxic potential. It should be noted that in cases where it is necessary from a feasibility standpoint to conduct the assays with the drug substance containing, or spiked with, the impurity, the proposed acceptance criterion should be commensurate with the level of impurity observed in clinical, stability, and/or production batches, taking into consideration the manufacturing and analytical variability. This acceptance criterion should not exceed the level present in the drug batch used in the genotoxicity assay and should be supported by the relevant qualification thresholds discussed in the ICH guidances or supporting general toxicity information.

In some cases, the structure of an impurity leading to the structural alert is shared with the API. The genotoxic potential of such an impurity can be evaluated through the standard testing of the API if the chemical environment for the alerting structure of the compounds is deemed comparable for the reactivity potential.

IV. RECOMMENDED APPROACHES FOR HANDLING GENOTOXIC AND CARCINOGENIC IMPURITIES

Positive results in one or more genotoxicity assays or other information indicating a carcinogenic potential, such as positive data from a carcinogenicity study with the impurity, should be addressed further. Recommended approaches for handling genotoxic or carcinogenic impurities are described in this section and are summarized in Table 2 at the end of section IV.C. A decision tree is also included in Appendix A.

A. Prevention of Genotoxic and Carcinogenic Impurity Formation

Since drug-related impurities presumably provide limited, if any, therapeutic benefits and because of their potential to cause cancer in humans, every feasible technical effort should be made to prevent the formation of genotoxic or carcinogenic compounds during drug substance synthesis or drug product manufacturing. However, we recognize that completely preventing the formation of or removing an impurity of concern may not be possible in many cases.

B. Reduction of Genotoxic and Carcinogenic Impurity Levels

In lieu of completely preventing the formation of a genotoxic or carcinogenic impurity, steps to reduce the level of impurity present in the drug substance or drug product should be considered. The following sections discuss acceptable thresholds to support safety during clinical development and for a marketing application. Analytical methodologies should be used that can adequately identify impurities of concern at levels associated with the relevant qualification thresholds. This threshold approach should be applied only in the absence of adequate qualification data (data that establish the biological safety of an impurity at the level specified) for the given impurity.

1. Acceptable Levels to Support Marketing Applications

In general, an exposure level of 1.5 µg per person per day for each impurity can be considered an acceptable qualification threshold for supporting a marketing application. Any impurity found at a level below this threshold generally should not need further safety qualification for genotoxicity and carcinogenicity concerns. The threshold is an estimate of daily exposure expected to result in an upper bound lifetime risk of cancer of less than 10^{-6} (one in a million), a risk level that is thought to pose negligible safety concerns. The threshold was based on an analysis of the carcinogenic potencies of 477 chemicals and was derived from the probability distribution of carcinogenic potencies of those compounds.[11] Subsequent analyses of an

[11] Fiori, JM and RD Meyerhoff, 2002, Extending the Threshold of Regulation Concept: De Minimis Limits for Carcinogens and Mutagens, Reg Toxicol Pharmacol, 35, 209-216.

298 expanded carcinogenic potency database of more than 700 carcinogens further confirmed the
299 threshold.[12] An additional analysis of subsets of highly potent carcinogens suggested that a
300 threshold of 0.15 µg per day, corresponding to a 10^{-6} lifetime risk of cancer, may be more
301 appropriate for chemicals with structural alerts for potential genotoxicity.[13] However, there are
302 some compounds containing certain structural groups (aflatoxin-like-, N-nitroso-, and azoxy-
303 structures) that have extremely high carcinogenic potency and are excluded from the threshold
304 approach.
305
306 Federal regulatory agencies in the United States, such as the Environmental Protection Agency
307 (EPA) (in the context of ambient water quality criteria), typically use a 10^{-6} lifetime risk of
308 cancer to determine *negligible* risk from chemical exposures.[14] This approach supports an
309 acceptable threshold level for genotoxic or carcinogenic impurities of 0.15 µg per day.
310 However, other regulatory bodies have proposed a 10^{-5} level as an acceptable cancer risk.[15,16]
311 Given that there is an overriding expected benefit of an approved drug product, a daily exposure
312 level of 1.5 µg per day, associated with a 10^{-5} lifetime risk of cancer, can be acceptable for most
313 genotoxic or carcinogenic impurities for a marketing application. This level of exposure is
314 expected to produce a negligible increase in carcinogenic risk based on the existing background
315 rate of human cancer and the conservative nature of cancer risk assessments. Additionally, this
316 threshold is considered to be low enough to ensure that the presence of a compound with an
317 uncharacterized genotoxic or carcinogenic potential would not significantly alter the risk-benefit
318 ratio of a drug product, even if the impurity is later shown to be a carcinogen.
319
320 The database from which the exposure threshold for genotoxic or carcinogenic impurities is
321 derived includes studies that primarily use oral administration, though a smaller number use the
322 inhalation route. Although the recommended threshold approach applies to all drug products
323 regardless of the intended route of administration, the qualification threshold of 1.5 µg per day
324 may not be appropriate for some routes (e.g., dermal, ophthalmic) because of the lack of a
325 relevant database from which an exposure threshold can be derived. Applicants should contact
326 specific drug review divisions regarding acceptable approaches in these cases.
327
328 As part of this threshold approach, applicants can conduct and provide to the FDA an SAR
329 assessment to identify structural similarities to known carcinogens. In cases where significant
330 structural similarities to a known carcinogen are identified, an estimate of the potential human

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[12] Ibid.

[13] Kroes, R, AG Renwick, M Cheeseman, J Kleiner, I Mangelsdorf, A Piersma, B Schilter, J Schlatter, F Schothorst, JG Vos, and G Würtzen, 2004, Structure-Based Threshold of Toxicological Concern (TTC): Guidance for Application to Substances Present at Low Levels in the Diet, Food Chem Toxicol, 42, 65-83.

[14] U.S. Environmental Protection Agency, Office of Water and Office of Science and Technology, 2000, Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health, document number EPA-822-B-00-004, section 1.5.3 (http://www.epa.gov/waterscience/humanhealth/method/complete.pdf).

[15] See EMEA guideline, section 5.2.3.

[16] World Health Organization Guidelines for Drinking-Water Quality, 2nd ed., Vol. 2, 1996, Health Criteria and Other Supporting Information, Geneva, World Health Organization, section 12.4.2 (http://www.who.int/water_sanitation_health/dwq/gdwq2v1/en/index1.html).

cancer risk can be calculated based on the available information for the confirmed carcinogen. This assessment can result in an increase in the acceptable exposure threshold for impurities that are highly similar to carcinogens with relatively low potency, or a reduction in the limit for impurities that are highly similar to relatively potent carcinogens.

The EPA guidance *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (EPA/630/R-03/003F) regarding cancer susceptibility in pediatric populations indicates that children exposed to mutagenic carcinogens between age 0 (birth) and 16 have an increased cancer risk over a 70-year lifetime when compared to adults.[17] EPA concludes that cancer risks generally are higher from early-life exposure than from similar exposure durations later in life and recommends the application of adjustment factors to risk calculations to account for this observation. EPA recommends an adjustment factor of 10 for exposures before 2 years of age (i.e., spanning a 2-year time interval from the first day after birth up until a child's second birthday), which represents an approximation of the weighted geometric mean tumor incidence ratio from juvenile or adult exposures in repeated dosing studies. In the absence of data to calculate a specific dose-response adjustment factor for exposures between 2 and less than 16 years of age, EPA recommends an adjustment factor of 3, which represents an intermediate level of adjustment and reflects a midpoint between the 10-fold adjustment for the first two years of life and no adjustment (i.e., 1-fold) for adult exposures. However, the EPA guidance acknowledges that the resultant increases in cancer risk are relatively small for exposures that continue with fair uniformity over a lifetime. We recommend that this increase in susceptibility to carcinogens in pediatric populations be considered when determining the acceptable impurity level for a given drug product.

The threshold approach for genotoxic or carcinogenic impurities limits the likelihood that any individual impurity in a given drug product will present more than a 10^{-5} excess cancer risk, but the approach is not intended to ensure an aggregate excess cancer risk of less than 10^{-5}. This means the threshold approach to individual impurities is not intended to limit the overall excess cancer risk to 10^{-5} from all impurities in a single drug product or from multiple drug products concomitantly administered. As discussed above, this approach is consistent with approaches taken by various regulatory bodies such as EPA, World Health Organization, and EMEA in implementing threshold levels for carcinogenic risk when no benefit from the expected exposure is perceived. However, in cases where a class or family of structurally similar impurities is identified and is expected to have similar mechanisms resulting in their genotoxic or carcinogenic potential, the total daily exposure to the related compounds should be evaluated relative to the recommended threshold exposure.

We recognize that drug products are often indicated for short-term use. However, for most drugs, these threshold considerations still apply since a drug may be used multiple times by the same individual or may be used outside of its approved indication. A detailed rationale should be provided to the FDA to support limits higher than generally considered appropriate for a marketing application.

[17] See http://cfpub.epa.gov/ncea/index.cfm.

2. Acceptable Levels during Clinical Development

The previous section describes the qualification threshold for genotoxic or carcinogenic impurities in support of a marketing application. Issues related to genotoxic impurities also can arise during a drug product's clinical development period and can affect the assessment of safety for conducting the program. Some flexibility in the previously described threshold level can be applied during the investigational stages, since clinical trials vary widely in duration from short-term (single dose to 4 weeks) to years and the qualification threshold for a marketing application is based on lifetime risk estimates. On the other hand, it should be recognized that during early clinical development, a benefit of the drug cannot be assumed. We recognize that the ability to identify and control drug-related impurities during early developmental stages is limited because of issues related to scale and maturity of production processes. Taking all these considerations into account, higher daily levels of exposure to potentially genotoxic impurities may be acceptable during the clinical development of the drug product compared to what is appropriate for a marketed drug product.

Bos et al. reviewed the derived cancer risk from short-term, high-dose exposure to a genotoxic carcinogen relative to the same cumulative dose distributed over a lifetime (virtually safe dose).[18] Briefly, the authors state that only a limited number of animal studies have assessed the comparative tumor incidence from short-term versus long-term exposures with similar cumulative doses. From those studies that do exist, dose rate correction factors (factors by which a specific dose of a chemical carcinogen at long-term, low-dose rates should be multiplied to derive the expected tumor incidence from short-term, high-dose rates) ranged from unity to 8.3. The authors conclude that the most pragmatic approach to calculate acceptable short-term exposures to known genotoxic carcinogens is to linearly extrapolate the short-term exposure from the acceptable lifetime exposure or virtually safe dose.

Acceptable daily intakes of genotoxic impurities during clinical development are presented in Table 1, based on the linear extrapolation approach described by Bos et al. The impurity threshold exposures for exposure durations of up to 12 months are based on a 10^{-6} cancer risk level (0.15 µg per day for a lifetime exposure), since these trials often include healthy subjects for whom there is no expected health benefit and the efficacy of the drug may still be uncertain. The values are derived from a linear extrapolation from the qualification threshold using the maximum duration of dosing for each time period specified in Table 1. In addition, these values incorporate an uncertainty factor of 2 to allow for deviations from the linear extrapolation model. For trials greater than 1-year duration, the threshold value is identical to the threshold for a marketing application and is based on a 10^{-5} cancer risk level (1.5 µg per day derived from lifetime exposures); subjects in these trials generally have the condition or disease being studied and are more certain to derive benefit from the treatment than subjects in early trials. When determining the acceptable impurity threshold exposure, the specifics of the patient population in the clinical trial should be evaluated.

[18] Bos, PMJ, B Baars, TM Marcel, and MTM van Raaij, 2004, Risk Assessment of Peak Exposure to Genotoxic Carcinogens: A Pragmatic Approach, Toxicol Letters, 151:43-50.

Table 1: Acceptable Qualification Thresholds for Genotoxic and Carcinogenic Impurities

	Duration of Clinical Trial Exposure					
	< 14 days	**14 days to 1 mo**	**1 mo to 3 mos**	**3 mos to 6 mos**	**6 mos to 12 mos**	**> 12 mos**
Genotoxic and carcinogenic impurity threshold (µg/day)	120	60	20	10	5	1.5

C. Additional Characterization of Genotoxic and Carcinogenic Risk

In cases where attempts to prevent the formation of an impurity of concern and/or to reduce the amount of the impurity to an acceptable level as per Table 1 are not possible, further characterization of the genotoxic and carcinogenic potential should be conducted. The guidance for industry and review staff *Recommended Approaches to Integration of Genetic Toxicology Study Results* describes the FDA's current thinking regarding appropriate additional evaluations that can be conducted.[19] Briefly, these concepts include the consideration of the mechanism of action, weight of evidence, or the conduct of additional supportive studies. These concepts also can be considered relevant for genotoxic impurities.

In addition to the above considerations, the conduct of an SAR evaluation of an impurity may provide useful information. When a significant structural similarity to a known carcinogen is identified, the drug substance and drug product acceptance criteria (typically in units of parts per million or percent) can be set at a level that is commensurate with the risk assessment specific to that of the known compound. As noted previously, the proposed factors should be considered in light of manufacturing batch data.

Table 2 summarizes the recommended approaches for characterizing the presence and addressing the safety of genotoxic and carcinogenic impurities depending on the clinical development stage.

[19] We update guidances periodically. To make sure you have the most recent version of a guidance, check the CDER guidance Web page at http://www.fda.gov/cder/guidance/index.htm.

Table 2: Recommended Approaches Based on Development Stage

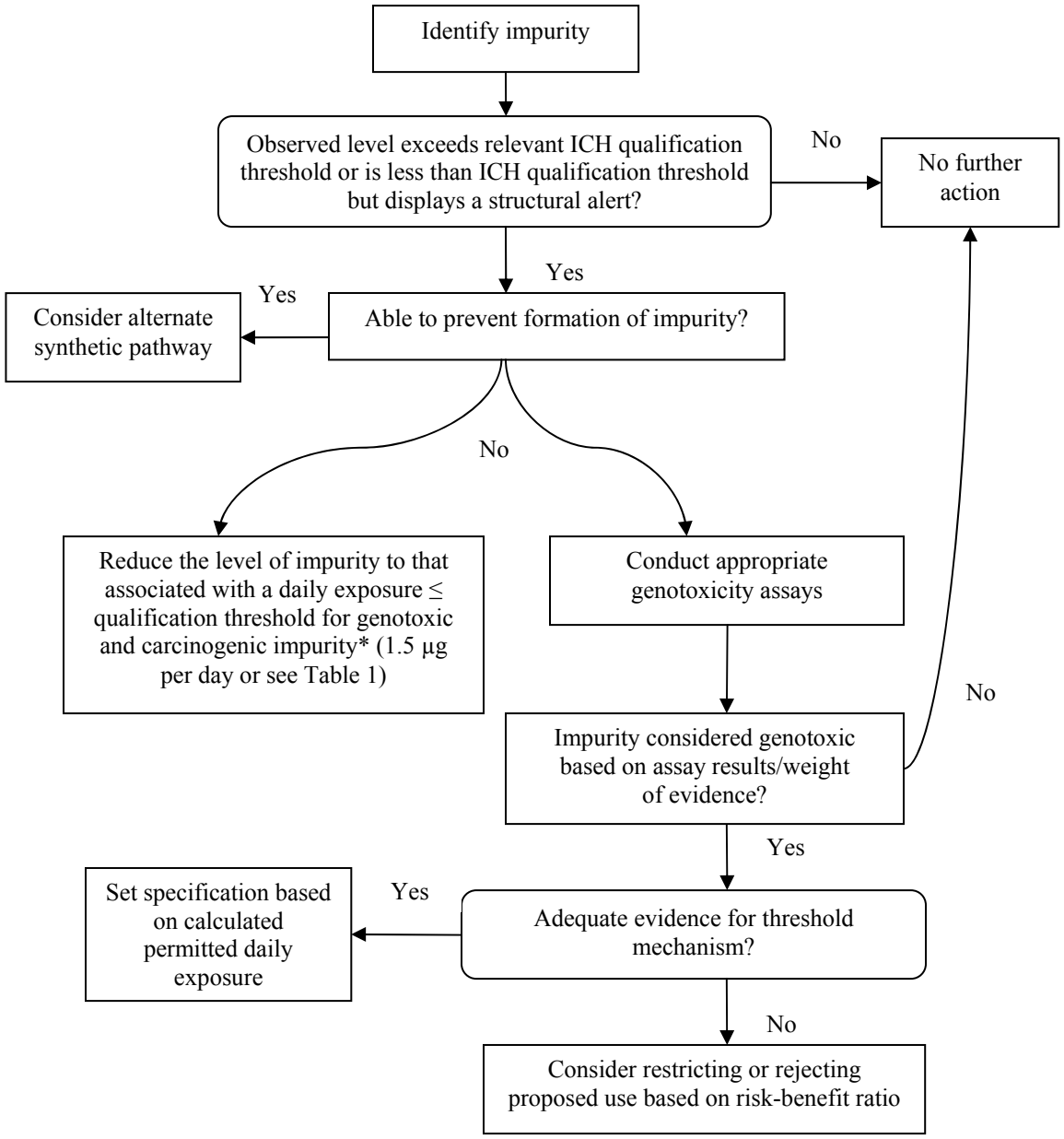
Clinical Development Stage	Recommended Approach
IND	• Evaluate identified impurities for genotoxic and carcinogenic risk via SAR assessment • Conduct assay for the presence of anticipated genotoxic and carcinogenic impurities • If impurity with genotoxic and carcinogenic potential is identified: – Modify synthetic pathway to eliminate the impurity, if possible OR – Conduct genotoxicity assays to characterize the genotoxic potential if not already known AND/OR – Set specification to that associated with a potential daily impurity exposure supported by compound-specific risk assessment or relevant qualification threshold (see Table 1)
Marketing application (NDA, BLA, or ANDA)	• Evaluate identified impurities for genotoxic and carcinogenic risk via SAR assessment • If impurity with genotoxic and carcinogenic potential is identified: – Conduct genotoxicity assays to characterize the genotoxic potential if not already known AND/OR – Set specification to that associated with a potential daily impurity exposure supported by compound-specific risk assessment or 1.5 µg per day threshold

D. Considerations for Flexibility in Approach

The previous sections are intended to be general recommendations to consider when developing a drug product in which a potentially genotoxic or carcinogenic impurity is identified. We recognize that these approaches may not necessarily apply to every development program, and flexibility in the application of these recommendations may be appropriate. When applying the recommendations, consideration should be given to the drug product's clinical development stage, the maximum duration of drug administration at that stage, the proposed indication (e.g., treatment of a life-threatening condition versus a less serious condition), the patient population (e.g., adults versus children), and the structural similarity of an impurity to a compound of known carcinogenic potency. In some of these cases, acceptance criteria higher than the recommended thresholds can be supported in the presence of a potential pharmacological benefit to patients. In rare cases, such as in the presence of highly potent carcinogens, decreases in the threshold also may be warranted. The appropriateness of a flexible approach should be informed by the feasibility of controlling impurity levels and the capabilities of the current process.

458 **APPENDIX A: DECISION TREE FLOW DIAGRAM**

459



460

*Safety threshold approach for genotoxic and carcinogenic impurities is not applicable to compounds with adequate data to derive compound-specific risk assessment or for those with SARs to high potency carcinogens. In addition, the approach may not be appropriate for some routes of administration (e.g., dermal, ophthalmic) because of the lack of a relevant database from which a threshold limit can be derived.

Exhibit 14

Concise International Chemical Assessment Document 31

N,*N*-DIMETHYLFORMAMIDE

Please note that the layout and pagination of this pdf file are not identical to those of the printed CICAD

First draft prepared by
G. Long and M.E. Meek, Environmental Health Directorate, Health Canada, and
M. Lewis, Commercial Chemicals Evaluation Branch, Environment Canada

Published under the joint sponsorship of the United Nations Environment Programme, the International Labour Organization, and the World Health Organization, and produced within the framework of the Inter-Organization Programme for the Sound Management of Chemicals.

World Health Organization
Geneva, 2001

The **International Programme on Chemical Safety (IPCS)**, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO), and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessment of the risk to human health and the environment from exposure to chemicals, through international peer review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The **Inter-Organization Programme for the Sound Management of Chemicals (IOMC)** was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research, and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

Concise International Chemical Assessment Documents (CICADs) are the latest in a family of publications from the International Programme on Chemical Safety (IPCS) — a cooperative programme of the World Health Organization (WHO), the International Labour Organization (ILO), and the United Nations Environment Programme (UNEP). CICADs join the Environmental Health Criteria documents (EHCs) as authoritative documents on the risk assessment of chemicals.

International Chemical Safety Cards on the relevant chemical(s) are attached at the end of the CICAD, to provide the reader with concise information on the protection of human health and on emergency action. They are produced in a separate peer-reviewed procedure at IPCS. They may be complemented by information from IPCS Poison Information Monographs (PIM), similarly produced separately from the CICAD process.

CICADs are concise documents that provide summaries of the relevant scientific information concerning the potential effects of chemicals upon human health and/or the environment. They are based on selected national or regional evaluation documents or on existing EHCs. Before acceptance for publication as CICADs by IPCS, these documents undergo extensive peer review by internationally selected experts to ensure their completeness, accuracy in the way in which the original data are represented, and the validity of the conclusions drawn.

The primary objective of CICADs is characterization of hazard and dose–response from exposure to a chemical. CICADs are not a summary of all available data on a particular chemical; rather, they include only that information considered critical for characterization of the risk posed by the chemical. The critical studies are, however, presented in sufficient detail to support the conclusions drawn. For additional information, the reader should consult the identified source documents upon which the CICAD has been based.

Risks to human health and the environment will vary considerably depending upon the type and extent of exposure. Responsible authorities are strongly encouraged to characterize risk on the basis of locally measured or predicted exposure scenarios. To assist the reader, examples of exposure estimation and risk characterization are provided in CICADs, whenever possible. These examples cannot be considered as representing all possible exposure situations, but are provided as guidance only. The reader is referred to EHC 170[1] for advice on the derivation of health-based tolerable intakes and guidance values.

While every effort is made to ensure that CICADs represent the current status of knowledge, new information is being developed constantly. Unless otherwise stated, CICADs are based on a search of the scientific literature to the date shown in the executive summary. In the event that a reader becomes aware of new information that would change the conclusions drawn in a CICAD, the reader is requested to contact IPCS to inform it of the new information.

Procedures

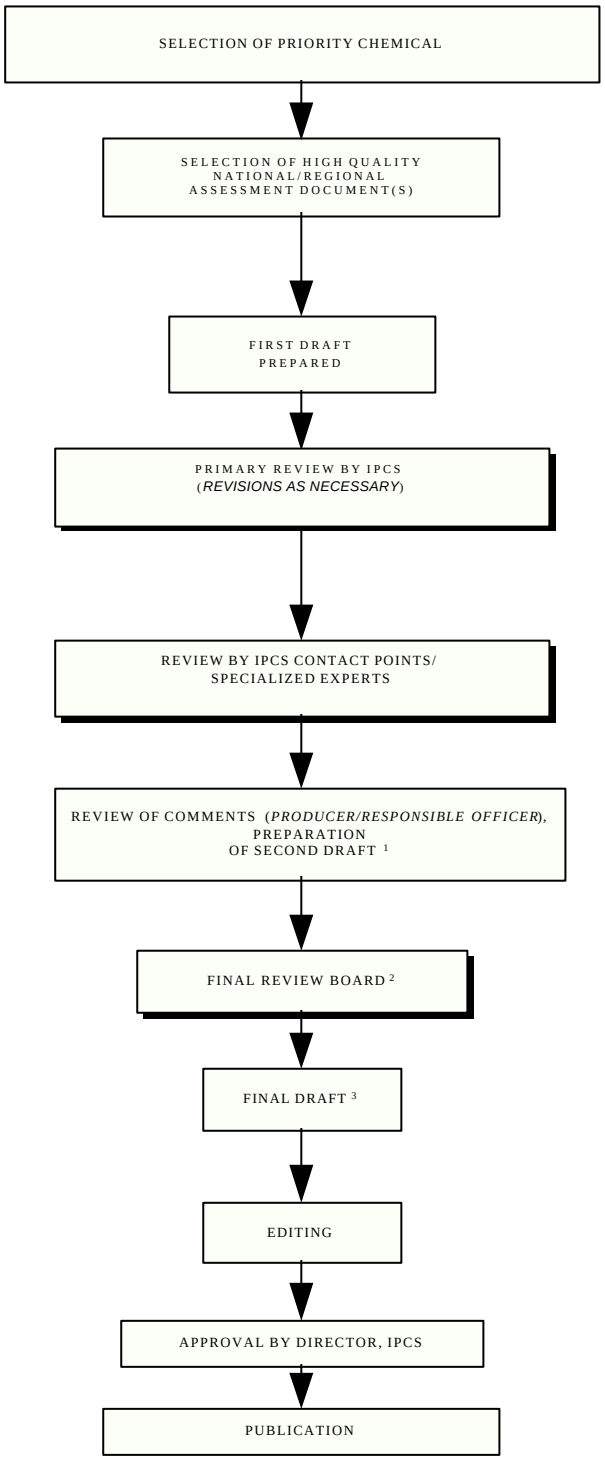
The flow chart shows the procedures followed to produce a CICAD. These procedures are designed to take advantage of the expertise that exists around the world — expertise that is required to produce the high-quality evaluations of toxicological, exposure, and other data that are necessary for assessing risks to human health and/or the environment. The IPCS Risk Assessment Steering Group advises the Co-ordinator, IPCS, on the selection of chemicals for an IPCS risk assessment, whether a CICAD or an EHC is produced, and which institution bears the responsibility of the document production, as well as on the type and extent of the international peer review.

The first draft is based on an existing national, regional, or international review. Authors of the first draft are usually, but not necessarily, from the institution that developed the original review. A standard outline has been developed to encourage consistency in form. The first draft undergoes primary review by IPCS and one or more experienced authors of criteria documents in order to ensure that it meets the specified criteria for CICADs.

The draft is then sent to an international peer review by scientists known for their particular expertise and by scientists selected from an international roster compiled by IPCS through recommendations from IPCS national Contact Points and from IPCS Participating Institutions. Adequate time is allowed for the selected experts to undertake a thorough review. Authors are required to take reviewers' comments into account and revise their draft, if necessary. The resulting second draft

[1] International Programme on Chemical Safety (1994) *Assessing human health risks of chemicals: derivation of guidance values for health-based exposure limits.* Geneva, World Health Organization (Environmental Health Criteria 170).

CICAD PREPARATION FLOW CHART



1 Taking into account the comments from reviewers.
2 The second draft of documents is submitted to the Final Review Board together with the reviewers' comments.
3 Includes any revisions requested by the Final Review Board.

is submitted to a Final Review Board together with the reviewers' comments.

A consultative group may be necessary to advise on specific issues in the risk assessment document.

The CICAD Final Review Board has several important functions:

– to ensure that each CICAD has been subjected to an appropriate and thorough peer review;
– to verify that the peer reviewers' comments have been addressed appropriately;
– to provide guidance to those responsible for the preparation of CICADs on how to resolve any remaining issues if, in the opinion of the Board, the author has not adequately addressed all comments of the reviewers; and
– to approve CICADs as international assessments.

Board members serve in their personal capacity, not as representatives of any organization, government, or industry. They are selected because of their expertise in human and environmental toxicology or because of their experience in the regulation of chemicals. Boards are chosen according to the range of expertise required for a meeting and the need for balanced geographic representation.

Board members, authors, reviewers, consultants, and advisers who participate in the preparation of a CICAD are required to declare any real or potential conflict of interest in relation to the subjects under discussion at any stage of the process. Representatives of nongovernmental organizations may be invited to observe the proceedings of the Final Review Board. Observers may participate in Board discussions only at the invitation of the Chairperson, and they may not participate in the final decision-making process.

1. EXECUTIVE SUMMARY

This CICAD on *N*,*N*-dimethylformamide (DMF) was prepared jointly by the Environmental Health Directorate of Health Canada and the Commercial Chemicals Evaluation Branch of Environment Canada based on documentation prepared concurrently as part of the Priority Substances Program under the *Canadian Environmental Protection Act* (CEPA). The objective of assessments on Priority Substances under CEPA is to assess potential effects of indirect exposure in the general environment on human health as well as environmental effects. Occupational exposure was not addressed in this source document. Data identified as of the end of September 1999 (environmental effects) and February 2000 (human health effects) were considered in this review. Information on the nature of the peer review and availability of the source document is presented in Appendix 1. Other reviews that were also consulted include IARC (1999) and BUA (1994). Information on the peer review of this CICAD is presented in Appendix 2. This CICAD was approved as an international assessment at a meeting of the Final Review Board, held in Helsinki, Finland, on 26–29 June 2000. Participants at the Final Review Board meeting are presented in Appendix 3. The International Chemical Safety Card (ICSC 0457) for *N*,*N*-dimethylformamide, produced by the International Programme on Chemical Safety (IPCS, 1999), has also been reproduced in this document.

N,*N*-Dimethylformamide (CAS No. 68-12-2) is an organic solvent produced in large quantities throughout the world. It is used in the chemical industry as a solvent, an intermediate, and an additive. It is a colourless liquid with a faint amine odour. It is completely miscible with water and most organic solvents and has a relatively low vapour pressure.

When emitted into air, most of the DMF released remains in that compartment, where it is degraded by chemical reactions with hydroxyl radicals. Indirect releases of DMF to air, such as transfers from other environmental media, play only a small role in maintaining levels of DMF in the atmosphere. DMF in air is estimated to be photooxidized over a period of days. However, some atmospheric DMF can reach the aquatic and terrestrial environment, presumably during rain events. When DMF is released into water, it degrades there and does not move into other media. When releases are into soil, most of the DMF remains in the soil — presumably in soil pore water — until it is degraded by biological and chemical reaction. Releases to water or soil are expected to be followed by relatively rapid biodegradation (half-life 18–36 h). If DMF reaches groundwater, its anerobic degradation will be slow. The use pattern of DMF is such that exposure of the general population is probably very low.

Since most DMF appears to be released to air in the sample country, and based on the fate of DMF in the ambient environment, biota are expected to be exposed to DMF primarily in air; little exposure to DMF from surface water, soil, or benthic organisms is expected. Based on this, and because of the low toxicity of DMF to a wide range of aquatic and soil organisms, the focus of the environmental risk characterization is terrestrial organisms exposed directly to DMF in ambient air.

DMF is readily absorbed following oral, dermal, or inhalation exposure. Following absorption, DMF is uniformly distributed, metabolized primarily in the liver, and relatively rapidly excreted as metabolites in urine. The major pathway involves the hydroxylation of methyl moieties, resulting in *N*-(hydroxymethyl)-*N*-methylformamide (HMMF), which is the major urinary metabolite in humans and animals. HMMF in turn can decompose to *N*-methylformamide (NMF). In turn, enzymatic *N*-methyl oxidation of NMF can produce *N*-(hydroxymethyl)formamide (HMF), which further degenerates to formamide. An alternative pathway for the metabolism of NMF is oxidation of the formyl group, resulting in *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC), which has been identified as a urinary metabolite in rodents and humans. A reactive intermediate, the structure of which has not yet been determined (possibly methyl isocyanate), is formed in this pathway; while direct supporting experimental evidence was not identified, this intermediate is suggested to be the putatively toxic metabolite. Available data indicate that a greater proportion of DMF may be metabolized by the putatively toxic pathway in humans than in experimental animals. There is metabolic interaction between DMF and alcohol, which, though not well understood, may be due, at least in part, to its inhibitory effect on alcohol dehydrogenase.

Consistent with the results of studies in experimental animals, available data from case reports and cross-sectional studies in occupationally exposed populations indicate that the liver is the target organ for the toxicity of DMF in humans. The profile of effects is consistent with that observed in experimental animals, with gastrointestinal disturbance, alcohol intolerance, increases in serum hepatic enzymes (aspartate aminotransferase, alanine aminotransferase, (-glutamyl transpeptidase, and alkaline phosphatase), and histopathological effects and ultrastructural changes (hepatocellular necrosis, enlarged Kupffer cells, microvesicular steatosis, complex

lysosomes, pleomorphic mitochondria, and fatty changes with occasional lipogranuloma) being observed.

Based on the limited data available, there is no convincing, consistent evidence of increases in tumours at any site associated with exposure to DMF in the occupational environment. Case reports of testicular cancers have not been confirmed in a cohort and case–control study. There have been no consistent increases in tumours at other sites associated with exposure to DMF.

There is also little consistent, convincing evidence of genotoxicity in populations occupationally exposed to DMF, with results of available studies of exposed workers (to DMF and other compounds) being mixed. The pattern of observations is not consistent with variations in exposure across studies. However, in view of the positive dose–response relationship observed in the one study in which it was investigated, this area may be worthy of additional work, although available data on genotoxicity in experimental systems are overwhelmingly negative.

DMF has low acute toxicity and is slightly to moderately irritating to the eyes and skin. No data were identified regarding the sensitization potential of DMF. In acute and repeated-dose toxicity studies, DMF has been consistently hepatotoxic, inducing effects on the liver at lowest concentrations or doses. The profile of effects includes alterations in hepatic enzymes characteristic of toxicity, increases in liver weight, progressive degenerative histopathological changes and eventually cell death, and increases in serum hepatic enzymes. A dose–response has been observed for these effects in rats and mice following inhalation and oral exposure. Species variation in sensitivity to these effects has been observed, with the order of sensitivity being mice > rats > monkeys.

Although the database for carcinogenicity is limited to two adequately conducted bioassays in rats and mice, there have been no increases in the incidence of tumours following chronic inhalation exposure to DMF. The weight of evidence for genotoxicity is overwhelmingly negative, based on extensive investigation in *in vitro* assays, particularly for gene mutation, and a more limited database *in vivo.*

In studies with laboratory animals, DMF has induced adverse reproductive effects only at concentrations greater than those associated with adverse effects on the liver, following both inhalation and oral exposure. Similarly, in well conducted and reported primarily recent developmental studies, fetotoxic and teratogenic effects have been consistently observed only at maternally toxic concentrations or doses.

Available data are inadequate as a basis for assessment of the neurological or immunological effects of DMF.

The focus of this CICAD and the sample risk characterization is primarily effects of indirect exposure in the general environment.

Air in the vicinity of point sources appears to be the greatest potential source of exposure of the general population to DMF. Based on the results of epidemiological studies of exposed workers and supporting data from a relatively extensive database of investigations in experimental animals, the liver is the critical target organ for the toxicity of DMF. A tolerable concentration of 0.03 ppm (0.1 mg/m^3) has been derived on the basis of increases in serum hepatic enzymes.

Data on the toxicity of DMF to terrestrial vascular plants have not been identified. Effect concentrations for indicators of the potential sensitivities of trees, shrubs, and other plants are high; hence, it is unlikely that terrestrial plants are particularly sensitive to DMF. For other terrestrial organisms, an estimated no-effects value of 15 mg/m^3 has been derived based on a critical toxicity value for hepatic toxicity in mice divided by an application factor. Comparison of this value with a conservative estimated exposure value indicates that it is unlikely that DMF causes adverse effects on terrestrial organisms in the sample country.

2. IDENTITY AND PHYSICAL/CHEMICAL PROPERTIES

N,*N*-Dimethylformamide (CAS No. 68-12-2) is a colourless liquid at room temperature with a faint amine odour (BUA, 1994). There are many synonyms for this compound, the most common being the acronym DMF. The molecular mass of DMF is 73.09, as calculated from its empirical formula (C_3H_7NO). DMF sold commercially contains trace amounts of methanol, water, formic acid, and dimethylamine (BUA, 1994).

DMF is miscible in all proportions with water and most organic solvents (Syracuse Research Corporation, 1988; Gescher, 1990; BUA, 1994; SRI International, 1994). DMF is also a powerful solvent for a variety of organic, inorganic, and resin products (SRI International, 1994). At temperatures below 100 °C, DMF

Table 1: Physical and chemical properties of DMF.

Property	Value	Reference	Values used in fugacity calculations[a]
Molecular mass	73.09		73.09
Vapour pressure (Pa at 25 °C)	490	Riddick et al. (1986)	490
Solubility (g/m³)	miscible	BUA (1994)	1.04×10^6
Log K_{ow}	!1.01	Hansch et al. (1995)	!1.01
Henry's law constant (Pa•m³/mol at 25 °C)	0.0345 0.0075	Bobra[b] BUA (1994)	0.034 53[c]
Density/specific gravity (g/ml at 25 °C)	0.9445	WHO (1991)	
Melting point (°C)	!60.5	WHO (1991)	!60.5 °C
Boiling point (°C)	153.5	WHO (1991)	
Half-life in air (h)	approx. 192	estimated from propane	170
Half-life in water (h)	18 36	Dojlido (1979) Ursin (1985)	55
Half-life in soil (h)	assumed to be equivalent to that in water		55
Half-life in sediment (h)	–		170
Half-life in suspended sediment (h)	–		55
Half-life in fish (h)	–		55
Half-life in aerosol (h)	–		5
Odour threshold	0.12–60 mg/m³	WHO (1991)	

[a] Discussed in section 11.1.3, Sample risk characterization.

[b] Collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

[c] Based upon vapour liquid equilibrium data (Hala et al., 1968), as calculated in DMER & AEL (1996).

remains stable in relation to light and oxygen (BUA, 1994). Temperatures in excess of 350 °C are required for DMF to decompose into carbon monoxide and dimethylamine (Farhi et al., 1968).[1]

Some important physical and chemical properties of DMF are summarized in Table 1. A vapour pressure of 490 Pa was recommended by Riddick et al. (1986). Because DMF is a miscible compound, it is preferable to determine the Henry's law constant experimentally. However, no experimental data were identified in the literature, and the calculated Henry's law constant of DMF remains uncertain (DMER & AEL, 1996).[2] The octanol/water partition coefficient (K_{ow}) was determined by a shake flask experiment (Hansch et al., 1995).

The conversion factor for DMF in air is as follows (WHO, 1991): 1 ppm = 3 mg/m^3.

3. ANALYTICAL METHODS

The following information on analytical methods for the determination of DMF in workplace air and biological media has been derived from WHO (1991) and Environment Canada (1999a).

3.1 DMF in workplace air

Colorimetric methods (based on the development of a red colour after the addition of hydroxylamine chloride as alkaline solution) that have often been utilized in the past are not specific (Farhi et al., 1968). Methods of choice more recently are high-performance liquid chromatography (HPLC) or gas chromatography – mass spectrometry (GCMS). Lauwerys et al. (1980)

[1] Also notes from N.J. Bunce, University of Guelph, Guelph, Ontario, to A. Chevrier, Environment Canada, 1 June 1998.

[2] Also collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

described a simple spectrophotometric method for measuring DMF vapour concentrations. Gas–liquid chromatography (GLC) is now the method of choice (Kimmerle & Eben, 1975a; NIOSH, 1977; Muravieva & Anvaer, 1979; Brugnone et al., 1980; Muravieva, 1983; Stransky, 1986). Detector tubes, certified by the US National Institute for Occupational Safety and Health, or other direct-reading devices calibrated to measure DMF (Krivanek et al., 1978; NIOSH, 1978) can be used. HPLC analysis (Lipski, 1982) can also be used. Mass spectrometric analysis for DMF in expired air has been described by Wilson & Ottley (1981), with a lower limit of detection of 0.5 mg/m^3. Figge et al. (1987) reported determination in air involving the enrichment of an organic polymer, thermal desorption of the adsorbed species, and qualitative determination by GCMS. The lower limit of detection was 5 ng/m^3. A NIOSH (1994) gas chromatographic (GC) method has an estimated detection limit of 0.05 mg per sample.

3.2 DMF and metabolites in biological media

DMF is extensively absorbed through the skin, its metabolism and kinetics are well known, and urinary metabolites exist that can be accurately measured. As a result, biological monitoring has been extensively used in the assessment of the absorbed amounts in occupationally exposed populations. The metabolite most often analysed is *N*-methylformamide (NMF), and several GC methods exist (Ikeda, 1996). Using nitrogen-sensitive detection, the limit of detection is 0.1 mg/litre.

4. SOURCES OF HUMAN AND ENVIRONMENTAL EXPOSURE

4.1 Natural sources

BUA (1994) identified no known natural sources of DMF. However, DMF is a possible product of the photochemical degradation of dimethylamine and trimethylamine (Pellizzari, 1977; Pitts et al., 1978; US EPA, 1986). Both are commonly occurring natural substances and are also used in industrial applications (European Chemicals Bureau, 1996a, 1996b).

4.2 Anthropogenic sources

Identified data on releases are restricted to the country of origin of the source document (Canada). They are presented here in the context of an example of an emissions profile.

In 1996, just over 16 tonnes of DMF were released from various industrial locations in Canada, of which 93% (15 079 kg) were emitted to the atmosphere and the remainder to water (245 kg), wastewater (204 kg), landfill sites (26 kg), or deep-well injection (669 kg) (Environment Canada, 1998). The Canadian market for DMF is quite small, with an estimated domestic consumption in the range of less than 1000 tonnes/year (SRI International, 1994; Environment Canada, 1998). The petrochemical sector was responsible for 84% (12.7 tonnes) of the reported atmospheric releases. Releases from the pharmaceutical industry accounted for 87% (0.212 tonnes) of total releases to water. Total release volumes from Canadian industrial sectors include 13.3 tonnes from the petrochemical sector, 1.2 tonnes from manufacture of pharmaceuticals, 0.7 tonnes from dye and pigment manufacture, 0.6 tonnes from polyvinyl chloride coating operations, 0.1 tonnes from its use as a solvent in pesticide manufacture, 0.07 tonnes from paint/finisher and paint remover manufacture, and 0.09 tonnes from other miscellaneous industrial sectors. For 1996, a reported total quantity of 0.056 tonnes was released (0.023 tonnes to air, 0.033 tonnes to water) by the producer during chemical synthesis of DMF (Environment Canada, 1998). Less than 1 tonne of DMF was released from wastewater treatment facilities and in landfills (Environment Canada, 1998). With a few exceptions, most industries reported little to no seasonal variation in releases (Environment Canada, 1998).

In the USA, between 23 and 47 million kilograms of DMF were produced in 1990 (US EPA, 1997).

World production of DMF is estimated to be 125 000 tonnes (Marsella, 1994).

The total consumption of DMF in Western Europe in 1989 was reported to be 55 000 tonnes (BUA, 1994). The production capacity was estimated to be 60 000 and 19 000 tonnes in the former Federal Republic of Germany and German Democratic Republic, respectively, 16 000 tonnes in Belgium, 15 000 tonnes in England, and 5000 tonnes in Spain (BUA, 1994).

Although small accidental releases (e.g., leakage of a storage tank or spill from a barrel) may remain unreported, available information suggests that spills of DMF during use, storage, or transport are not a significant route of entry to the environment (Environment Canada, 1999a).

The quantity of DMF in landfill sites should be small. The total quantity of DMF used in formulation of products (other than pesticides) appears to be small in comparison to its use as a manufacturing aid, cleaner, or degreaser (Environment Canada, 1998). As such,

consumer products deposited in landfill sites should contain little or no DMF. The industrial DMF deposited directly in landfill sites consists only of residues remaining after incineration (Environment Canada, 1998).

4.3 Uses

DMF is used commercially as a solvent in vinyl resins, adhesives, pesticide formulations, and epoxy formulations; for purification and/or separation of acetylene, 1,3-butadiene, acid gases, and aliphatic hydrocarbons; and in the production of polyacrylic or cellulose triacetate fibres and pharmaceuticals (WHO, 1991; IARC, 1999). DMF is also used in the production of polyurethane resin for synthetic leather (Fiorito et al., 1997).

5. ENVIRONMENTAL TRANSPORT, DISTRIBUTION, AND TRANSFORMATION

5.1 Air

The atmospheric pathway is particularly important in determining exposure to DMF. This is due to the fact that industrial releases of DMF into air appear to be considerably larger than releases to other environmental media (BUA, 1994; Environment Canada, 1998).

Because of the complete miscibility of DMF in water, atmospheric DMF may be transported from air into surface water or soil pore water during rain events (DMER & AEL, 1996).[1] Atmospheric DMF should be present in the vapour phase and therefore should be readily available for leaching out by rainfall (US EPA, 1986).[2] Although the efficiency and rate of washout are unknown, precipitation events (i.e., rain, snow, fog) likely shorten the residence time of DMF in the atmosphere. As water has an atmospheric half-life of approximately 4 days at Canadian latitudes, this can be considered the minimum atmospheric half-life of DMF in relation to precipitation.[1]

Chemical degradation of DMF in air is likely due to reaction with hydroxyl radicals (Hayon et al., 1970). The possibility of photochemical decomposition (i.e., direct photolysis) of DMF is extremely small (Grasselli, 1973; Scott, 1998). Other chemical degradation processes — for example, reaction with nitrate radicals — are not known to significantly affect the fate of DMF in air.

The reaction rate constant (k_{OH}) for the formamide functional group is unknown. However, the degradation half-life of DMF can be roughly estimated by comparing DMF with other compounds in terms of their relative atmospheric reactivity.

Based on experiments in chambers, reactivity for DMF relative to propane is low (Sickles et al., 1980). The k_{OH} of propane is 1.2×10^{-12} cm^3/molecule per second (Finlayson-Pitts & Pitts, 1986). Using the global average hydroxyl radical concentration of 7.7×10^5 molecules/cm^3 (Prinn et al., 1987) and the calculation method proposed by Atkinson (1988), the half-life of propane is estimated at approximately 8 days.

Although the degradation half-life of DMF in air cannot be estimated with certainty, the available evidence therefore suggests that the half-life is at least 8 days (192 h). The mean half-life used for fugacity-based fate modelling was 170 h, as it is frequently used to represent a half-life range of 100–300 h (DMER & AEL, 1996). This half-life may be underestimated; however, sensitivity analysis on the fugacity-based results indicates that percent partitioning estimates are not sensitive to this parameter, but estimated concentrations are affected.[3]

5.2 Surface water and sediment

Once released into surface water, DMF is unlikely to transfer to sediments, biota, or the atmosphere. With a K_{ow} of !1.01 (Hansch et al., 1995), DMF remains in the dissolved form and is not expected to adsorb to the organic fraction of sediments or suspended organic matter. This K_{ow} also suggests that DMF does not concentrate in aquatic organisms (BUA, 1994); indeed, no bioaccumulation was observed in carp during an 8-week bioaccumulation test (Sasaki, 1978). With a Henry's law constant of 0.0345 Pa@m^3/mol, volatilization from water is expected to be slight (BUA, 1994).[3]

The overall rate of chemical degradation is expected to be very slow in surface water.

[1] Also letter from D.R. Hastie, York University, Toronto, Ontario, to P. Doyle, Environment Canada, 1998.

[2] Also technical note from N.J. Bunce, University of Guelph, Guelph, Ontario, to B. Scott, Environment Canada, dated 10 February 1998.

[3] Collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

Photochemical decomposition is unlikely in water (Grasselli, 1973; US EPA, 1986). The photooxidation half-life of DMF in water was estimated experimentally at 50 days and would be even longer in the natural environment where other compounds compete for reaction with hydroxyl radicals (Hayon et al., 1970). The rate of hydrolysis of amides like DMF at normal temperatures in laboratory studies is extremely slow, even under strong acid or base conditions (Fersht & Requena, 1971; Eberling, 1980). The low temperature (generally less than 20 °C) and near-neutral pH of natural surface water therefore limit and almost preclude the hydrolysis of DMF under normal environmental conditions (Frost & Pearson, 1962; Langlois & Broche, 1964; Scott, 1998).

Biodegradation appears to be the primary degradation process in surface water. Under experimental conditions, DMF was degraded, either aerobically or anaerobically, by various microorganisms and algae in activated sludges, over a wide range of concentrations (Hamm, 1972; Begert, 1974; Dojlido, 1979). Intermediate biodegradation products include formic acid and dimethylamine, which further degrade to ammonia, carbon dioxide, and water (Dojlido, 1979; Scott, 1998). In some studies, acclimation periods of up to 16 days preceded quantitative degradation (Chudoba et al., 1969; Gubser, 1969). Extended adaptation under specific experimental conditions may also account for negative degradation results observed in a few studies with incubation times #14 days (Kawasaki, 1980; CITI, 1992). Limited degradation was reported in seawater (range 1–42%) (Ursin, 1985), and no degradation was found after 8 weeks' incubation under anaerobic conditions (Shelton & Tiedje, 1981).

Biodegradation of DMF in receiving surface waters is unlikely to be affected by the inherent toxicity of DMF and its biodegradation products. Concentrations above 500 mg/litre in effluent reduced the efficiency of treatment systems using activated sludge (Thonke & Dittmann, 1966; Nakajima, 1970; Hamm, 1972; Begert, 1974; Carter & Young, 1983). However, even with continuous releases, such high concentrations of DMF are not anticipated in natural waters.

In a river die-away test, an initial concentration of 30 mg DMF/litre completely disappeared within 3 and 6 days from unacclimated and acclimated water, respectively (Dojlido, 1979). The mineralization rate of DMF in seawater was less than 3% in 24 h for initial concentrations of 10 µg/litre and 100 µg/litre. However, 20% was mineralized in 24 h at a concentration of 0.1 µg/litre (Ursin, 1985). A half-life of 55 h was used for water in the fugacity-based fate modelling described in section 5.4 (DMER & AEL, 1996).[1,2] No information is available on the half-life of DMF in sediments. DMER & AEL (1996) recommend a half-life in sediment of 170 h based on the assumption that reactivity in sediment is slower than in soil.

5.3 Soil and groundwater

Fugacity-based fate modelling and the miscibility of DMF indicate that some of the DMF released into the atmosphere can reach the ground, in part, at least, through rainfall (DMER & AEL, 1996).[1,2] Once in soils, DMF will be degraded by chemical and biological processes or leached into groundwater.

As rain fills the available pore space in soils, DMF is incorporated into the pore water. With an octanol/water partition coefficient of !1.01 (Hansch et al., 1995), DMF will not tend to adsorb to humic material. Weak bonds with the mineral phase are possible but likely insignificant because of the high solubility of DMF.[3]

Biological degradation and, to a lesser extent, chemical processes operating in surface water would also likely affect DMF contained in soil pore water (Scott, 1998). As for surface water, biodegradation should therefore be the primary breakdown mechanism in soils. A soil bacterial culture acclimated to small amounts of petroleum and petroleum products degraded DMF under aerobic conditions within 18 h (Romadina, 1975), indicating a soil biodegradation half-life similar to the one observed in water. A somewhat longer conservative half-life of 55 h was used in fugacity-based fate modelling (DMER & AEL, 1996).[1,2]

The miscibility of DMF and its low Henry's law constant indicate limited volatilization from moist soils (BUA, 1994). However, DMF will be efficiently removed from soils by leaching into groundwater, likely at the same speed as water percolates through the soil.[4] This is

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[1] Also technical note sent from R. Beauchamp, Health Canada, to A. Chevrier, Environment Canada, 1998.

[2] Also collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

[3] Letter from K. Bolton, University of Toronto, Toronto, Ontario, to A. Chevrier, Environment Canada, dated 8 June 1998.

[4] Technical note from S. Lesage to B. Elliott, Environment Canada, dated 26 November 1997.

supported by a calculated organic carbon/water partition coefficient (K_{oc}) of 7 (Howard, 1993) and a soil sorption coefficient (K_{om}) of about 50, estimated from quantitative structure–activity relationships (Sabljic, 1984; US EPA, 1986), which both indicate that DMF is mobile in soils. If it reaches groundwater, DMF will be slowly degraded anaerobically (Scott, 1998).[1]

5.4 Environmental distribution

Fugacity modelling was conducted to provide an overview of key reaction, intercompartment, and advection (movement out of a system) pathways for DMF and its overall distribution in the environment. A steady-state, non-equilibrium model (Level III fugacity modelling) was run using the methods developed by Mackay (1991) and Mackay & Paterson (1991). Assumptions, input parameters, and results are summarized in Environment Canada (1999a) and presented in detail in DMER & AEL (1996) and by Beauchamp[2] and Bobra.[3] Modelling predictions do not reflect actual expected concentrations in the environment but rather indicate the broad characteristics of the fate of the substance in the environment and its general distribution among the media.

Modelling results identify air as an important exposure medium. If DMF is emitted into air, fugacity modelling predicts that 61% of the chemical will be present in air, 32% in soil, and only 7% in water. These results suggest that most of the DMF released into air will remain in that compartment, where it will be degraded by chemical reactions. They also indicate that some atmospheric DMF can reach the aquatic and terrestrial environment — presumably in rain and runoff (Scott, 1998).[4] However, the quantity of DMF available for entrainment in rain and runoff is limited by degradation in the atmosphere.

Fugacity modelling also indicates that when DMF is continuously discharged into either water or soil, most of it can be expected to be present in the receiving medium. For example, if it is released into water, 99% of the DMF is likely to be present in the water, and subsequent transport into sediment or bioconcentration in biota is not likely to be significant. When releases are into soil, 94% of the material remains in the soil — presumably in soil pore water (Scott, 1998). Therefore, indirect releases of DMF to air, such as transfers from other environmental media, play only a small role in maintaining levels of DMF in the atmosphere.

It is important to note that fugacity-based partitioning estimates are significantly influenced by input parameters such as the Henry's law constant, which, in this case, is highly uncertain. Therefore, the above partitioning estimates are also uncertain.

6. ENVIRONMENTAL LEVELS AND HUMAN EXPOSURE

6.1 Environmental levels

6.1.1 Ambient air

Concentrations of DMF in stack emissions of two Canadian industries were less than 7.5 mg/m^3 (Environment Canada, 1998, 1999b). Data on concentrations in ambient air around these sources are not available.

In Lowell, Massachusetts, USA (Amster et al., 1983), DMF was detected in the air over an abandoned chemical waste reclamation plant (0.007 mg/m^3), a neighbouring industry (>0.15 mg/m^3), and a residential area (0.024 mg/m^3). Ambient air samples collected in the northeastern USA in 1983 ranged from less than 0.000 02 to 0.0138 mg DMF/m^3 (Kelly et al., 1993, 1994). In samples taken in 1983, levels of DMF were generally less than 0.02 mg/m^3 at a hazardous waste site in unsettled wind conditions, possibly as high as 9 mg/m^3 at nearby industrial sites, and less than 0.02 mg/m^3 in adjoining residential areas (Clay & Spittler, 1983).

A range of 0.000 11 – 0.0011 mg/m^3 was reported in Japan in 1991, but specific locations and proximity to sources were not provided (Environment Agency Japan, 1996). In Germany, a concentration of $0.005 µg DMF/m^3 was detected in air (Figge et al., 1987).

[1] Technical note from S. Lesage to B. Elliott, Environment Canada, dated 26 November 1997.

[2] Technical note sent from R. Beauchamp, Health Canada, to A. Chevrier, Environment Canada, 1998.

[3] Collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

[4] Also letter from S. Lei, Atomic Energy Control Board of Canada, to A. Chevrier, Environment Canada, dated 11 June 1998.

6.1.2 Surface water and sediment

DMF was detected (detection limit 0.002 mg/litre) in only 1 of 204 surface water samples collected between August 1975 and September 1976 from 14 heavily industrialized river basins in the USA (Ewing et al., 1977). The Environment Agency Japan (1996) reported concentrations between 0.0001 and 0.0066 mg/litre in 18 out of 48 water samples taken in 1991. In addition, in 24 water samples collected in 1978, levels were below the detection limits of 0.01–0.05 mg/litre (Environment Agency Japan, 1985). The proximity of these measurements to industrial sources is not known.

In Canada, monitoring data are available for effluents at one southern Ontario location, which released less than ~0.03 tonnes into surface water in 1996 (Environment Canada, 1998). The facility reported a range of <1–10 mg DMF/litre in effluents, but has since established a wastewater treatment plant, which reduced its effluent concentrations to non-detectable levels (detection limit 0.5 mg/litre). DMF was detected in 1 of 63 industrial effluents in the USA at a detection limit of approximately 0.01 mg/litre (Perry et al., 1979). The US Environmental Protection Agency (EPA)[1] also cited an effluent concentration of 0.005 mg/litre at a sewage treatment plant in 1975.

The properties of DMF and fugacity modelling indicate negligible accumulation of DMF in sediments (BUA, 1994; Hansch et al., 1995; DMER & AEL, 1996).[2,3] However, concentrations of 0.03–0.11 mg/kg were reported in sediments (9 out of 48 samples) in Japan (Environment Agency Japan, 1996). No information was provided on proximity to sources of DMF, sediment characteristics, or hydrological regimes. In addition, because information on sampling and analytical methods was not provided, the quality of these data cannot be assessed. In 24 sediment samples collected in 1978 at unspecified locations in Japan, levels were below the detection limits of 0.1–0.3 mg/kg (Environment Agency Japan, 1985).

6.1.3 Soil and groundwater

In 3 of 23 groundwater samples collected in the USA, concentrations ranged from 0.05 to 0.2 mg/litre, with an average value of 0.117 mg/litre (Syracuse Research Corporation, 1988).[1]

6.2 Human exposure

6.2.1 Drinking-water

Although DMF was listed as a contaminant in a survey of drinking-water in the USA, quantitative data were not reported (Howard, 1993).

6.2.2 Food

Data on concentrations of DMF in foods were not identified.

6.2.3 Multimedia study

A Health Canada-sponsored multimedia exposure study for DMF and other volatile organic compounds was conducted in 50 homes in the Greater Toronto Area in Ontario, Nova Scotia, and Alberta (Conor Pacific Environmental, 1998). DMF was not detected in indoor air samples from the 50 residences (detection limit 3.4 µg/m^3). It was also not detected in tap water samples, although the limit of detection was high (0.34 µg/ml). DMF was not recovered reproducibly in composite food or beverage samples in this study.

6.2.4 Exposure of the general population

Identified data on concentrations of DMF in environmental media in Canada were insufficient to allow estimates of population exposure to be developed; for water, either quantitative data on concentrations are unreliable[4] or DMF has not been detected, using analytical methodology with poor sensitivity (Conor Pacific Environmental, 1998).

Non-pesticidal use of DMF in Canada is small and restricted primarily to industrial applications. Most DMF released into the environment in Canada during such use is emitted to air. Most DMF remains in the medium of release prior to degradation. Therefore, the greatest potential for exposure of the general population to DMF

[1] Group STORET search on DMF, obtained from J. Boyd, US EPA (storet@epamail.eap.gov), on 30 July 1999.

[2] Also technical note sent from R. Beauchamp, Health Canada, to A. Chevrier, Environment Canada, 1998.

[3] Also collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

[4] Technical notes regarding data from Environmental Monitoring and Reporting Branch, Ontario Ministry of Environment and Energy, sent to J. Sealy, Health Canada, 1996.

from non-pesticidal sources is in air in the vicinity of industrial point sources.

Based upon dispersion modelling of releases in Canada from the highest emitter over a 1-km radius, 100 m in height, the estimated ambient concentration is 110 µg/m^3. Although this value is comparable to levels measured under similar conditions in other countries, it is based on very conservative assumptions; taking into account more likely conditions, including some loss due to advection, estimated concentrations would be 10- to 100-fold less (i.e., 11 or 1.1 µg/m^3).

Based on lack of detection in a multimedia study, levels of DMF in indoor air of 50 homes in Canada were less than 3.4 µg/m^3 (Conor Pacific Environmental, 1998).

6.2.5 Occupational exposure

Occupational exposure to DMF may occur in the production of the chemical itself, other organic chemicals, resins, fibres, coatings, inks, and adhesives (IARC, 1999). Exposure may also occur during use of these coatings, inks, and adhesives in the synthetic leather industry, in the tanning industry, and as a solvent in the repair of aircraft (Ducatman et al., 1986; IARC, 1989).

Based on data from the National Exposure Data Base, maintained by the United Kingdom Health and Safety Executive, concentrations of DMF in workplace air in the manufacture of textiles ranged from 0.1 to 10.5 ppm (0.3 to 7.5 mg/m^3) in 16 facilities.[1] For the six facilities where data were reported, the 8-h time-weighted average (TWA) concentration ranged from 4 to 12.4 ppm (12 to 37.2 mg/m^3). At six facilities where plastic was manufactured, concentrations ranged from 0.1 to 0.7 ppm (0.3 to 2.1 mg/m^3). At 11 facilities for plastics processing, the range of concentrations was from 4 to 44 ppm (12 to 132 mg/m^3); the range of 8-h threshold limit values (TLVs) at six of the facilities was 5–38 ppm (15–114 mg/m^3).

In the USA between 1981 and 1983, approximately 125 000 workers were potentially exposed to DMF, with 13 000 workers potentially exposed for more than 20 h/week (NIOSH, 1983).

[1] Data retrieval by J. Tickner from National Exposure Data Base, Health and Safety Executive (hse.gsi.gov.uk), 2000.

7. COMPARATIVE KINETICS AND METABOLISM IN LABORATORY ANIMALS AND HUMANS

Available data indicate that DMF is readily absorbed following oral, dermal, and inhalation exposure in both humans and animals. The rate of dermal absorption was estimated to be 57 mg/cm^2 per 8 h in a rat tail model. DMF is metabolized primarily in the liver and is relatively rapidly excreted as metabolites in urine, primarily as *N*-(hydroxymethyl)-*N*-methylformamide (HMMF).

7.1 Experimental animals

The major metabolic pathway for DMF in mammalian species is oxidation by the cytochrome P-450-dependent mixed-function oxidase system to HMMF (Figure 1). This can generate NMF and formaldehyde (see review by Gescher, 1993). Further cytochrome P-450-mediated oxidation of NMF and/or HMMF results in the formation of *S*-(*N*-methylcarbamoyl)glutathione (SMG), the conjugate of the presumed reactive (toxic) intermediate, methyl isocyanate, excreted *in vivo* as *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC). Results of studies with liver microsomes from acetone-treated rats (Mráz et al., 1993; Chieli et al., 1995) and mice (Chieli et al., 1995) and with reconstituted enzyme systems indicate that cytochrome P-450 2E1 mediates the metabolism of DMF to HMMF and, subsequently, to the proposed reactive intermediate, methyl isocyanate.

The most informative of the toxicokinetic and metabolic studies relevant to consideration of interspecies and dose-related variations in toxicokinetics and metabolism include investigations following oral administration to rats and inhalation exposure of rats, mice, and monkeys.[2]

In female Sprague-Dawley rats administered a single oral dose of 100 mg ^{14}C-labelled DMF/kg body weight on day 12 or 18 of pregnancy, 60–70% of the radioactivity was excreted in urine and 3–4% in faeces at 48 h (Saillenfait et al., 1997). Approximately 4% of the dose was present in the liver at 0.5 h after dosing at both gestation times, with 8 and 13% in the gastrointestinal tract (stomach and intestine) and 0.7 and 0.8% in

[2] In early studies, HMMF was not reported, since it degraded to NMF thermolytically in GLC conditions; hence, in early investigations, NMF = HMMF + NMF. HMMF is stable in aqueous solutions of neutral or mildly acidic pH but undergoes thermal decomposition to NMF during routine GC analysis. Therefore, it was first identified as NMF.

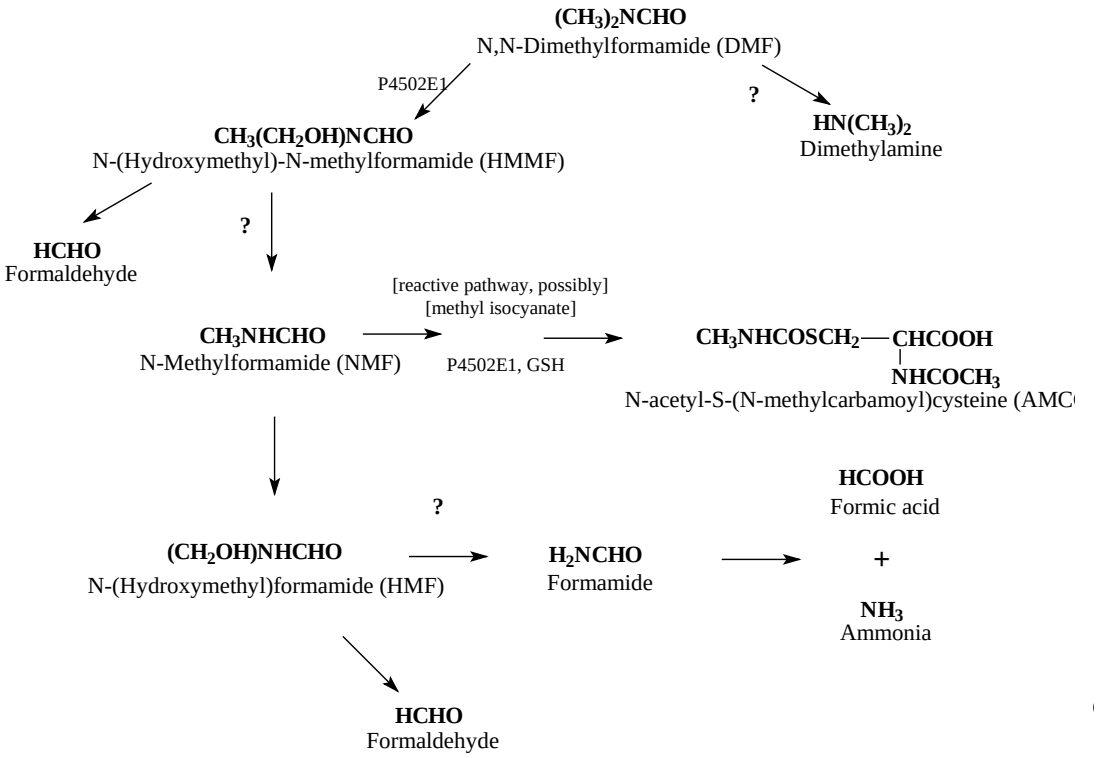

Fig. 1. Biotransformation of DMF (adapted from WHO, 1991; Gescher, 1993).

the kidneys, respectively. Plasma radioactivity was relatively constant from 0.5 to 4 h after dosing (approximately 0.4–0.5% of the dose) but declined rapidly thereafter. By 48 h, only the liver (0.5 and 0.6%) and intestine (0.2 and 0.3%) retained any significant activity. In animals exposed on day 12 of gestation, approximately 1.5% of the dose was present in the uterus, placenta, embryo, and amniotic fluid at between 0.5 and 4 h, which rapidly declined to less than 0.1% at 24 h. In rats exposed on day 18 of gestation, fetal tissues accounted for 6% of the administered dose. HPLC analysis performed at intervals from 1 to 24 h indicated that unchanged DMF and metabolites were readily transferred to the embryonic and fetal tissues, where levels were generally equal to those in maternal plasma. The parent compound accounted for most of the radioactivity until 4–8 h and then decreased.

Levels of parent compound and metabolites were determined in the plasma, amniotic fluid, placenta, and embryo in this investigation. Unchanged DMF initially accounted for the major proportion of radiolabelled carbon in the plasma or tissues, 61–77% for the first 4 h and 73–93% for the first 8 h after treatment on days 12 and 18, respectively. The decline in DMF levels corresponded with an increase in the levels of HMMF and NMF. HMMF accounted for 40–47% of ^{14}C at 8 h (day 12) and for 41–55% at 16 h (day 18). The equivalent figures for NMF were 9–13% and 16–18%, respectively. The amounts of AMCC and formamide in plasma or tissues were <4% of total radioactivity at all time points (Saillenfait et al., 1997). Other investigators have reported that DMF also crosses the placenta of pregnant rats after inhalation exposure (Sheveleva et al., 1977; Shumilina, 1991).

In another of the few recent investigations, levels of DMF, NMF, and HMMF were determined in the blood and urine of B6C3F1 mice and Crl:CD BR rats exposed to 10, 250, or 500 ppm (30, 750, or 1500 mg/m^3) for either single exposures of 1, 3, or 6 h or for 6 h/day, 5 days/week, for 2 weeks (Hundley et al., 1993a). The values for area under the plasma concentration curve (AUC) for DMF increased disproportionately in comparison with exposure, following single 6-h exposures to 250 and 500 ppm (750 and 1500 mg/m^3) (8- and 28-fold for rats and mice, respectively), while levels of NMF in the blood did not increase, which the authors considered to be indicative of saturation of metabolism of DMF. In contrast, multiple exposures increased the capacity of both rats and mice to metabolize DMF; repeated exposures to 500 ppm (1500 mg/m^3) resulted in a 3- and 18-fold reduction in AUC values for rats and mice, respectively. Peak plasma levels for NMF were elevated. HMMF represented over 90% of the total of DMF and determined metabolites.

In a similar investigation, DMF, NMF, and HMMF in blood and urine were determined in male and female cynomolgus monkeys exposed to 30, 100, or 500 ppm (90, 300, or 1500 mg/m^3) for 6 h/day, 5 days/week, for 13 weeks (Hundley et al., 1993b). The values for the AUC increased disproportionally between 100 and 500 ppm (300 and 1500 mg/m^3) (19- to 37-fold in males and 35- to 54-fold in females), data consistent with saturation of metabolism. However, there was no corresponding decrease in NMF levels; rather, they increased proportionally with increases in exposure concentrations. For each concentration, AUC values, peak plasma concentration, and plasma half-lives were consistent throughout the duration of exposure. HMMF was the main urinary metabolite (56–95%), regardless of exposure level or duration of exposure. DMF was not readily excreted in the urine, and NMF was more prevalent in plasma than in urine, suggesting that it was metabolized to compounds not determined in the study.

In comparative analyses of the two studies, the authors indicated that toxicokinetic differences may, in part, contribute to the observed species differences in toxicity. The AUC values and peak plasma levels for DMF for rats and mice following a single 500 ppm (1500 mg/m^3) exposure are substantially greater than the respective values in monkeys following a similar exposure. Whereas repeated exposures to 500 ppm (1500 mg/m^3) in rats and mice enhanced metabolism, as indicated by diminished AUC values for DMF and increased plasma concentrations of NMF, this effect was not clearly demonstrated in monkeys.

Results of the more recent study in rats were qualitatively similar to earlier investigations in which plasma DMF and "NMF" levels were determined in the plasma of rats exposed to DMF by inhalation for single 3- or 6-h exposures (Kimmerle & Eben, 1975a; Lundberg et al., 1983). Results of several of these earlier studies were also suggestive that at very high concentrations, DMF inhibits its own biotransformation. For example, 3 h following a single 4-h inhalation exposure of rats to 1690 or 6700 mg/m^3, levels of NMF in blood were lower in the higher exposure group (Lundberg et al., 1983). Similarly, Kimmerle & Eben (1975a) reported lower concentrations of NMF in the blood of rats exposed to 6015 mg/m^3 for 3 h than in rats exposed to 513 mg/m^3 for 6 h.

In a number of early studies, the effects of co-administration of ethanol on blood concentrations of DMF, NMF, ethanol, and acetaldehyde were investigated. Although there were variations in results depending on dose, time interval between administration of DMF and ethanol, and routes of exposure, there were increases in concentrations of DMF, NMF, ethanol, or acetaldehyde in blood upon co-exposure. These results may be attributable to inhibition by DMF of the activity of alcohol dehydrogenase observed both *in vitro* and *in vivo* (Eben & Kimmerle, 1976; Hanasono et al., 1977; Sharkawi, 1979) and of aldehyde dehydrogenase observed *in vivo* (Elovaara et al., 1983).

7.2 Humans

7.2.1 Studies in human volunteers

There were a number of early investigations in which the parent compound and some metabolites (not including that of the putatively toxic pathway) in blood and urine were determined in volunteers following short-term exposure to DMF (26 or 87 ppm [78 or 261 mg/m^3] for 4 h or 4 h/day for 5 days) (Kimmerle & Eben, 1975b). Results of these investigations indicated that DMF was rapidly excreted (the majority in 24 h), primarily as HMMF. Results of an additional early study in volunteers indicated that co-exposure to ethanol had a "slight influence" on the metabolism of DMF in volunteers receiving 19 g of ethanol 10 min prior to exposure to 82 ppm (246 mg/m^3) DMF for 2 h, based on lower concentrations of NMF in blood upon co-exposure. Contrary to the results in animals, there were no significant differences in the blood levels of ethanol and acetaldehyde upon co-exposure, which the authors attributed to the relatively low concentrations of DMF (Eben & Kimmerle, 1976).

In a recent study in which the product of the putatively toxic pathway of metabolism (AMCC) was determined, 10 volunteers were exposed to 10, 30, or 60 mg DMF/m^3, for either single 8-h exposures or five daily exposures of 30 mg/m^3 (Mráz & Nohová, 1992a, 1992b). Urine was collected for 5 days and analysed for DMF, HMMF, HMF, and AMCC. In a separate protocol, three volunteers ingested 20 mg AMCC dissolved in water, and metabolites were determined for a period of 8 h after exposure. After single exposure to 30 mg/m^3, the proportions of metabolites eliminated in the urine were 0.3% parent compound, 22.3% HMMF, 13.2% HMF, and 13.4% AMCC. The half-times of excretion for these various metabolites were approximately 2, 4, 7, and 23 h, respectively. In contrast to this slow elimination after exposure to DMF, AMCC was rapidly eliminated after ingestion of AMCC, with a half-time of 1 h. These results were considered to be consistent with rate-limiting reversible protein binding of a reactive metabolic intermediate of DMF, possibly methyl isocyanate. Following repeated exposures, AMCC accumulated in urine. Although quantitative data were not presented, urinary elimination 16 h following the fifth exposure was approximately 14% HMMF, 32% HMF, and 54% AMCC.

7.2.2 Occupational environment

Exposure in the occupational environment may occur through both the dermal and inhalation routes. Lauwerys et al. (1980) reported that dermal absorption was more important than inhalation in the overall exposure, in the absence of personal protective devices.

There have been a number of reports of levels of DMF and metabolites in the blood and/or urine of workers. With the exception of more recent studies involving personal air sampling (Wrbitzky & Angerer, 1998),[1] few provide reliable quantitative data on relationship with exposure, though still not accounting for additional dermal exposure. Results of such studies have confirmed, however, the presence of AMCC (the product of the putatively toxic metabolic pathway) in the urine of workers.

Wrbitzky & Angerer (1998) noted a weak association between the concentration of DMF in workplace air and urinary concentration of NMF. Kawai et al. (1992) considered the relationship to be linear. In 116 workers exposed to TWA concentrations of 0.2, 0.4, 0.6, 3.9, or 9.1 ppm (0.6, 1.2, 1.8, 11.7, or 27.3 mg/m^3), the corresponding concentrations of NMF in urine were 0.7, 0.9, 2.6, 7.8, and 19.7 mg/litre.

Mráz et al. (1989) reported the detection of HMMF in urine samples from 12 DMF-exposed workers (extent of exposure not specified). Casal Lareo & Perbellini (1995) reported that AMCC accumulated throughout the work week in the urine of workers exposed to approximately 3–8 ppm (9–24 mg/m^3). Sakai et al. (1995) reported that levels of urinary AMCC remained constant over consecutive work days and increased after the end of exposure, with the peak concentration observed at 16–40 h after the end of exposure. Kafferlein[1] reported that urinary NMF concentrations were highest in post-shift samples, with a median half-time of 5.1 h. Concentrations of urinary AMCC reached a steady state 2 days after the beginning of exposure, with a half-time greater than 16 h.

7.2.3 Other relevant data

Angerer et al. (1998) reported that haemoglobin from individuals occupationally exposed to DMF contained *N*-carbamoylated valine residues derived from methyl isocyanate, the likely precursor of AMCC. The metabolism of DMF to HMMF by human liver microsomes *in vitro* has also been demonstrated. The addition of an antibody against rat liver cytochrome P-450 2E1 to the incubation mixture strongly inhibited DMF metabolism (Mráz et al., 1993).

7.3 Interspecies comparisons

In one of the few identified studies in which the product of the putatively toxic metabolic pathway (i.e., AMCC) was determined in animal species, Mráz et al. (1989) reported data on metabolites of DMF (DMF, HMMF, "HMF," AMCC) in 72-h urine samples following intraperitoneal administration of 0.1, 0.7, or 7 mmol/kg body weight to mice, rats, and hamsters. In addition, 10 healthy volunteers (5 males, 5 females) were exposed for 8 h to 20 ppm (60 mg/m^3). (The mean of the amount of DMF absorbed via the lung was reported to be half of the lowest dose administered in rodents.) Urine was collected and analysed for the same metabolites at 2- to 8-h intervals for 8 h for 4–5 days. The proportion of the total metabolites eliminated as AMCC was greatest in the rat (1.7–5.2%) and less in the hamster (1.5–1.9%) and mouse (1.1–1.6%). In rats exposed to the highest dose, excretion of DMF metabolites (including AMCC) was delayed. There was no clear dose-related variation in proportion of the metabolites determined excreted as AMCC in the animal species. In humans, a greater proportion of the absorbed dose (14.5%) following

[1] Also written comments provided by H. Kafferlein, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander University Erlangen-Nuremberg, Germany, 2000.

inhalation was present as AMCC in the urine. Absorption through the skin was not taken into account.

8. EFFECTS ON LABORATORY MAMMALS AND *IN VITRO* TEST SYSTEMS

8.1 Single exposure

Following oral, dermal, inhalation, or parenteral administration, the acute toxicity of DMF in a number of species is low. Lethal doses are generally in the g/kg body weight range for oral, dermal, and parenteral routes and in the g/m^3 range for inhalation exposure. Clinical signs following acute exposure include general depression, anaesthesia, loss of appetite, loss of body weight, tremors, laboured breathing, convulsions, haemorrhage at nose and mouth, liver injury, and coma preceding death. Where protocols included histopathological examination, damage was observed primarily in the liver (WHO, 1991). In the rat, oral LD_{50}s range from 3000 to 7170 mg/kg body weight, dermal LD_{50}s range from 5000 to >11 520 mg/kg body weight, and inhalation LC_{50}s range from 9432 to 15 000 mg/m^3 (WHO, 1991).

8.2 Irritation and sensitization

Standard tests for dermal irritation by DMF have not been identified, and data on its sensitization potential are conflicting. Hence, only limited conclusions can be drawn concerning the potential of DMF to induce these effects.

IARC (1999), WHO (1991), and Kennedy (1986) reviewed the effects of DMF on the skin and eyes and reported only mild to moderate effects. A single application of neat DMF to the shaved skin of mice at 1–5 g/kg body weight (precise exposure conditions not specified) produced slight transient skin irritation at 2.5–5 g/kg body weight, while similar treatment of rabbits at up to 0.5 g/kg body weight was without effect (Kennedy, 1986; WHO, 1991). Repeated (15- or 28-day) applications of 1–2 g/kg body weight did not induce marked local effects on the skin of rats or rabbits. The instillation of neat or 50% aqueous DMF into the rabbit eye produced moderate corneal injury and moderate to severe conjunctivitis, with some damage still evident 14 days later (Kennedy, 1986; WHO, 1991; IARC, 1999).

In a murine local lymph node assay predictive for identification of contact allergens, cell proliferation (based on [^{3}H]thymidine incorporation in lymph nodes) was significantly increased (324 vs. 193 decompositions per minute per lymph node in exposed and control groups, respectively) in mice (strain not specified) receiving a daily topical application of 25 µl on the dorsum of both ears for 3 consecutive days (Montelius et al., 1996). In subsequent assays, thymidine incorporation in DMF-exposed mice was up to 3-fold higher than in naive mice. However, statistical analyses were not presented, and the increase was not considered to be significant (Montelius et al., 1998). The naive (non-treated) mice were included in the protocol to measure the magnitude of vehicle (DMF)-induced proliferation. In contrast, Kimber & Weisenberger (1989) detected no difference in proliferation in a lymph node assay in which lymph node cells from DMF (the solvent)-exposed mice were compared with those from naive mice.

8.3 Short-term exposure

While there have been a number of primarily early short-term studies, these have generally been restricted to examination of specific effects following exposure to single dose levels. They are not additionally informative concerning the toxicity of DMF but confirm a range of effects in the liver, which, when considered collectively across studies, are consistent with a profile in rats of alterations in hepatic enzymes and increases in liver weight at lowest concentrations and degenerative histopathological changes, cell death, and increases in serum hepatic enzymes at higher concentrations. Although results of a short-term study in monkeys also indicate that this species is less sensitive to the effects of DMF than rats, the protocol had only one exposure concentration, and there were only two monkeys in the experiment (Hurtt et al., 1991).

In the only short-term investigation in which a dose–response relationship for hepatic effects was characterized, there was a dose-related increase in liver to body weight ratio, significant at all levels of exposure, and in activity of uridine disphosphate glucuronosyltransferase in male Wistar rats exposed for 2 weeks via drinking-water to approximately 0, 14, 70, or 140 mg/kg body weight per day (Elovaara et al., 1983). Such changes have not been observed at such low doses in more recent, longer-term studies.

Available data from acute and short-term studies also indicate that there are effects on metabolizing enzymes at very high doses (i.e., 475 mg/kg body weight per day and above administered subcutaneously to rats). These include glutathione metabolism (although reported changes at two different doses were not

consistent) and decreases in hepatic microsomal P-450 content (Imazu et al., 1992, 1994; Fujishiro et al., 1996).

8.4 Medium-term exposure

Information on the incidences of lesions in the critical medium-term exposure studies is presented in Tables 2 and 3.

8.4.1 Inhalation

The NTP (1992a) carried out a subchronic bioassay in F344 rats, exposing males and females to 0, 50, 100, 200, 400, or 800 ppm (0, 150, 300, 600, 1200, or 2400 mg/m^3) for 6 h/day, 5 days/week, for 13 weeks. The authors designated 200 ppm (600 mg/m^3) as a no-observed-adverse-effect level (NOAEL) for both sexes, based upon the absence of histopathological lesions in liver. Minimal to moderate hepatocellular necrosis in both sexes was observed at 400 and 800 ppm (1200 and 2400 mg/m^3), with the lesion more severe in females. However, in males, both the absolute and relative weights of liver were significantly increased at 100 ppm (300 mg/m^3) and greater, although there was no clear dose–response, as weights declined at the highest dose. Serum cholesterol was increased at all levels of exposure; again, there was no clear dose–response. In males at day 24, there was a dose-related increase in serum alanine aminotransferase (ALT) (significant at all levels of exposure); however, at day 91, the increase was significant only at 400 ppm (1200 mg/m^3). At day 91, there was also a dose-related increase in serum sorbitol dehydrogenase in males (significant at 200 ppm [600 mg/m^3]). In females, relative liver weight was significantly increased at all levels of exposure, with the weight declining at the highest dose. Serum cholesterol was significantly increased at all levels of exposure in females, with no clear dose–response. At day 91 in females, serum sorbitol dehydrogenase and isocitrate dehydrogenase were significantly increased at 200 ppm (600 mg/m^3) and greater.

Craig et al. (1984) exposed male and female F344 rats to 0, 150, 300, 600, or 1200 ppm (0, 450, 900, 1800, or 3600 mg/m^3) for 6 h/day, 5 days/week, for 12 weeks. There were few overt signs of toxicity. Body weight was significantly decreased in both sexes at the highest dose. There were some changes in clinical chemistry and haematological parameters at the highest doses. In males, serum cholesterol was significantly increased at the highest concentration only. Serum alkaline phosphatase (AP) was reduced in a dose-related manner, beginning at 300 ppm (900 mg/m^3). In females, cholesterol was significantly increased at 600 and 1200 ppm (1800 and 3600 mg/m^3). In contrast to males, serum AP was increased in a dose-related manner (significant at the two highest concentrations). Data on organ weights were not presented. Histopathological changes were observed in the liver at the highest doses, were "barely discernible" at 300 ppm (900 mg/m^3), and were not observed at 150 ppm (450 mg/m^3). The lowest-observed-adverse-effect concentration (LOAEC) for both sexes is 300 ppm (900 mg/m^3), based upon slight histopathological changes in the liver (no-observed-effect concentration [NOEC] = 150 ppm [450 mg/m^3]).

B6C3F1 mice were exposed to 0, 50, 100, 200, 400, or 800 ppm (0, 150, 300, 600, 1200, or 2400 mg/m^3) for 6 h/day, 5 days/week, for 13 weeks (NTP, 1992a). Relative liver weight was significantly increased in both sexes at all levels of exposure, although the dose–response was not clear. Absolute liver weight was significantly increased in females at all dose levels, although the dose–response was not clear. Centrilobular hepatocellular hypertrophy (minimal to mild) was observed in all exposed males and in females at 100 ppm (300 mg/m^3) and higher (lowest-observed-effect concentration [LOEC] = 50 ppm [150 mg/m^3]).

Craig et al. (1984) exposed B6C3F1 mice to 0, 150, 300, 600, or 1200 ppm (0, 450, 900, 1800, or 300 mg/m^3) for 6 h/day, 5 days/week, for 12 weeks. Mortality was 10% at 600 ppm (1800 mg/m^3) and 40% at 1200 ppm (3600 mg/m^3). No adverse effects on haematology or clinical chemistry were observed. Hepatic cytomegaly was observed in all exposed mice; the incidence and severity were related to dose (LOEC = 150 ppm [450 mg/m^3]).

Hurtt et al. (1992) exposed three male and three female cynomolgus monkeys to 0, 30, 100, or 500 ppm (0, 90, 300, or 1500 mg/m^3) for 6 h/day, 5 days/week, for 13 weeks. Two males were maintained for a further 13-week observation period after exposure had ceased. The protocol included microscopic examination of a comprehensive range of organ tissues in all animals. Sperm morphology and vaginal cytology were also evaluated in all animals. There were no overt signs of toxicity and no effects on body weight gain, haematology, clinical chemistry, urinalysis, organ weights, or histopathological effects attributable to DMF in cynomolgus monkeys exposed to up to 500 ppm (1500 mg/m^3), leading the authors to conclude that the monkey is much less sensitive than the rat or mouse (Hurtt et al., 1992).

The other inhalation studies are either poorly reported or limited in their scope (Massmann, 1956; Clayton et al., 1963; Cai & Huang, 1979; Arena et al., 1982). One group of investigators reported effects on the

Table 2: Effect levels and benchmark concentrations for DMF, inhalation exposure.

Study (reference)	Effect level	Data for calculating benchmark concentration: Concentration	Response	Benchmark concentration: Parameter estimates[a,b]		Goodness of fit
Medium-term exposure						
B6C3F1 mice 10 males and 10 females per group 0, 50, 100, 200, 400, 800 ppm, 6 h/day, 5 days/week, for 13 weeks (NTP, 1992a)	LOEC = 50 ppm, based upon increased relative liver weight in both sexes and hepatocellular hypertrophy in males	Male, incidence (severity) of centrilobular hepatocellular hypertrophy: control 50 ppm 100 ppm 200 ppm 400 ppm 800 ppm	 0/10 4/10 (1.8) 9/10 (1.3) 10/10 (2.0) 10/10 (2.0) 10/10 (2.0)	BMC_{05} = 8.5 ppm excluding 400 and 800 ppm groups Adjusted BMC_{05} = 1.51 ppm	95% LCL_{05} = 2.5 ppm excluding 400 and 800 ppm groups Adjusted 95% LCL_{05} = 0.44 ppm	Chi-square (1) = 0.004 *P*-value = 0.99
		Female, incidence (severity) of centrilobular hepatocellular hypertrophy: control 50 ppm 100 ppm 200 ppm 400 ppm 800 ppm	 0/10 0/10 10/10 (1.3) 10/10 (1.9) 10/10 (2.0) 10/10 (2.0)	BMC_{05} = 17.9 ppm excluding 200, 400, and 800 ppm groups Adjusted BMC_{05} = 3.19 ppm excluding 200, 400, and 800 ppm groups	95% LCL_{05} = 8.1 ppm excluding 200, 400, and 800 ppm groups Adjusted 95% LCL_{05} = 1.45 ppm excluding 200, 400, and 800 ppm groups	Chi-square (1) = 7.5 *P*-value = 0.01
Long-term exposure/carcinogenicity assays						
Rat, Crl:CD BR 87 males and 87 females per group 0, 25, 100, 400 ppm, 6 h/day, 5 days/week, for 2 years (Malley et al., 1994)	LOEC = 100 ppm, based upon a signif-icant increase in centrilobular hepatocellular hypertrophy (both sexes), hepatic accumulation of lipo-fuscin/haemosiderin (both sexes), and hepatic single-cell necrosis (females only) NOEC = 25 ppm	females, hepatic accumulation of lipofuscin/haemosiderin: control (*n* = 60) 25 ppm (*n* = 59) 100 ppm (*n* = 59) 400 ppm (*n* = 62)	 8% 7% 22% (*P* < 0.05) 61% (*P* < 0.05)	BMC_{05} = 37.0 ppm Adjusted BMC_{05} = 6.61 ppm	95% LCL_{05} = 19.8 ppm Adjusted 95% LCL_{05} = 3.54 ppm	Chi-square (1) = 1.01 *P*-value = 0.31
		males, hepatic accumulation of lipofuscin/haemosiderin: control (*n* = 57) 25 ppm (*n* = 59) 100 ppm (*n* = 58) 400 ppm (*n* = 60)	 4% 4% 17% (*P* < 0.05) 58% (*P* < 0.05)	BMC_{05} = 41.4 ppm Adjusted BMC_{05} = 7.39 ppm	95% LCL_{05} = 21.9 ppm Adjusted 95% LCL_{05} = 3.91 ppm	Chi-square (1) = 0.84 *P*-value = 0.36
		males, relative liver weight: control (*n* = 17) 25 ppm (*n* = 19) 100 ppm (*n* = 21) 400 ppm (*n* = 26)	 2.87 2.81 3.28 3.58 (*P* < 0.05)	BMC_{05} = 44.5 ppm Adjusted BMC_{05} = 7.95 ppm	95% LCL_{05} = 23.7 ppm Adjusted 95% LCL_{05} = 4.23 ppm	F(1,79) = 2.09 *P*-value = 0.15
		males, hepatic foci of alterations (clear cell): control (*n* = 57) 25 ppm (*n* = 59) 100 ppm (*n* = 58) 400 ppm (*n* = 60)	 11% 8% 22% (*P* < 0.05) 35% (*P* < 0.05)	BMC_{05} = 57.7 ppm Adjusted BMC_{05} = 10.3 ppm	95% LCL_{05} = 37.8 ppm Adjusted 95% LCL_{05} = 6.75 ppm	Chi-square (2) = 1.71 *P*-value = 0.42

Table 2 (contd).

Study (reference)	Effect level	Data for calculating benchmark concentration: Concentration	Response	Benchmark concentration: Parameter estimates[a,b]		Goodness of fit
		females, hepatic foci of alterations (clear cell):		BMC_{05} = 84.3 ppm Adjusted BMC_{05} = 15.1 ppm	95% LCL_{05} = 53.4 ppm Adjusted 95% LCL_{05} = 9.54 ppm	Chi-square (2) = 0.77 *P*-value = 0.68
		control (*n* = 60)	5%			
		25 ppm (*n* = 59)	5%			
		100 ppm (*n* = 59)	14%			
		400 ppm (*n* = 62)	24% (*P* < 0.05)			
Rat, Crl:CD BR 87 males and 87 females per group 0, 25, 100, 400 ppm, 6 h/day, 5 days/week, for 2 years (Malley et al., 1994)	LOEC = 100 ppm, based upon a significant increase in centrilobular hepatocellular hypertrophy (both sexes), hepatic accumulation of lipofuscin/haemosiderin (both sexes), and hepatic single-cell necrosis (females only) NOEC = 25 ppm	females, relative liver weight:		BMC_{05} = 101.6 ppm Adjusted BMC_{05} = 18.1 ppm	95% LCL_{05} = 46.2 ppm Adjusted 95% LCL_{05} = 8.25 ppm	F(1,67) = 1.12 *P*-value = 0.29
		control (*n* = 22)	3.12			
		25 ppm (*n* = 14)	3.43			
		100 ppm (*n* = 12)	3.33			
		400 ppm (*n* = 23)	3.86 (*P* < 0.05)			
		males, centrilobular hepatocellular hypertrophy:		BMC_{05} = 118.7 ppm Adjusted BMC_{05} = 21.2 ppm	95% LCL_{05} = 56.4 ppm Adjusted 95% LCL_{05} = 10.1 ppm	Chi-square (1) = 0.65 *P*-value = 0.42
		control (*n* = 57)	0			
		25 ppm (*n* = 59)	0			
		100 ppm (*n* = 58)	5% (*P* < 0.05)			
		400 ppm (*n* = 60)	30% (*P* < 0.05)			
		females, centrilobular hepatocellular hypertrophy:		BMC_{05} = 126.7 ppm Adjusted BMC_{05} = 22.6 ppm	95% LCL_{05} = 77.7 ppm Adjusted 95% LCL_{05} = 13.9 ppm	Chi-square (1) = 0.13 *P*-value = 0.72
		control (*n* = 60)	0			
		25 ppm (*n* = 59)	0			
		100 ppm (*n* = 59)	3% (*P* < 0.05)			
		400 ppm (*n* = 62)	40% (*P* < 0.05)			
		females, hepatic single-cell necrosis:		BMC_{05} = 126.9 ppm Adjusted BMC_{05} = 22.7 ppm	95% LCL_{05} = 72.9 ppm Adjusted 95% LCL_{05} = 13.0 ppm	Chi-square (1) = 0.78 *P*-value = 0.38
		control (*n* = 60)	0			
		25 ppm (*n* = 59)	0			
		100 ppm (*n* = 59)	5% (*P* < 0.05)			
		400 ppm (*n* = 62)	18% (*P* < 0.05)			
Mice, Crl:CD 1 (ICR)BR 78 males and 78 females per group 0, 25, 100, 400 ppm, 6 h/day, 5 days/week, for 18 months (Malley et al., 1994)	LOEC = 25 ppm, based upon centrilobular hepatocellular hypertrophy (males), hepatic single-cell necrosis (males and females), and hepatic Kupffer cell hyperplasia/pigment accumulation (males)	females, hepatic single-cell necrosis:		BMC_{05} = 16.8 ppm BMC_{05} = 5.9 ppm excluding 400 ppm group Adjusted BMC_{05} = 3.00 ppm BMC_{05} = 1.05 ppm excluding 400 ppm group	95% LCL_{05} = 11.9 ppm 95% LCL_{05} = 4.1 ppm excluding 400 ppm group Adjusted 95% LCL_{05} = 2.13 ppm 95% LCL_{05} = 0.73 ppm excluding 400 ppm group	Chi-square (2) = 9.7 *P*-value = 0.00 (Chi-square (1) = 0.02 *P*-value = 0.88)
		control (*n* = 61)	29%			
		25 ppm (*n* = 63)	44% (*P* < 0.05)			
		100 ppm (*n* = 61)	70% (*P* < 0.05)			
		400 ppm (*n* = 63)	76% (*P* < 0.05)			
		males, hepatic single-cell necrosis:		BMC_{05} = 10.8 ppm Adjusted BMC_{05} = 1.93 ppm	95% LCL_{05} = 7.8 ppm Adjusted 95% LCL_{05} = 1.39 ppm	Chi-square (2) = 13.4 *P*-value = 0.00
		control (*n* = 60)	24%			
		25 ppm (*n* = 62)	59% (*P* < 0.05)			
		100 ppm (*n* = 60)	68% (*P* < 0.05)			
		400 ppm (*n* = 59)	87% (*P* < 0.05)			
		males, hepatic Kupffer cell hyperplasia/pigment accumulation:		BMC_{05} = 11.1 ppm Adjusted BMC_{05} = 1.98 ppm	95% LCL_{05} = 8.2 ppm Adjusted 95% LCL_{05} = 1.46 ppm	Chi-square (2) = 7.5 *P*-value = 0.02
		control (*n* = 60)	22%			
		25 ppm (*n* = 62)	52% (*P* < 0.05)			
		100 ppm (*n* = 60)	60% (*P* < 0.05)			
		400 ppm (*n* = 59)	86% (*P* < 0.05)			

Table 2 (contd).

Study (reference)	Effect level	Data for calculating benchmark concentration		Benchmark concentration		
		Concentration	Response	Parameter estimates[a,b]		Goodness of fit
		females, hepatic Kupffer cell hyperplasia/pigment accumulation:		BMC_{05} = 13.4 ppm Adjusted BMC_{05} = 2.39 ppm	95% LCL_{05} = 9.3 ppm Adjusted 95% LCL_{05} = 1.66 ppm	Chi-square (2) = 0.35 *P*-value = 0.84
		control (*n* = 61)	51%			
		25 ppm (*n* = 63)	57%			
		100 ppm (*n* = 61)	71% (*P* < 0.05)			
		400 ppm (*n* = 63)	89% (*P* < 0.05)			
Mice, Crl:CD 1 (ICR)BR 78 males and 78 females per group 0, 25, 100, 400 ppm, 6 h/day, 5 days/week, for 18 months (Malley et al., 1994)	LOEC = 25 ppm, based upon centrilobular hepatocellular hypertrophy (males), hepatic single-cell necrosis (males and females), and hepatic Kupffer cell hyperplasia/pigment accumulation (males)	males, centrilobular hepatocellular hypertrophy:		BMC_{05} = 18.9 ppm Adjusted BMC_{05} = 3.38 ppm BMC_{05} = 2.93 ppm excluding 400 ppm group	95% LCL_{05} = 15.3 ppm Adjusted 95% LCL_{05} = 0.95 ppm 95% LCL_{05} = 1.48 ppm excluding 400 ppm group	Chi-square (2) = 0.77 *P*-value = 0.00 (Chi-square (0) = 0.00 *P*-value = 1.00)
		control (*n* = 60)	0			
		25 ppm (*n* = 62)	8% (*P* < 0.05)			
		100 ppm (*n* = 60)	41% (*P* < 0.05)			
		400 ppm (*n* = 59)	52% (*P* < 0.05)			
		females, centrilobular hepatocellular hypertrophy:		BMC_{05} = 25.1 ppm Adjusted BMC_{05} = 4.48 ppm	95% LCL_{05} = 19.9 ppm Adjusted 95% LCL_{05} = 3.55 ppm	Chi-square (2) = 0.39 *P*-value = 0.82
		control (*n* = 61)	0			
		25 ppm (*n* = 63)	6%			
		100 ppm (*n* = 61)	19% (*P* < 0.05)			
		400 ppm (*n* = 63)	54% (*P* < 0.05)			
		males, relative liver weight:		BMC_{05} = 65.6 ppm Adjusted BMC_{05} = 11.7 ppm	95% LCL_{05} = 37.5 ppm Adjusted 95% LCL_{05} = 6.69 ppm	F(1,143) = 1.94 *P*-value = 0.17
		control (*n* = 31)	5.85			
		25 ppm (*n* = 42)	5.94			
		100 ppm (*n* = 38)	7.06 (*P* < 0.05)			
		400 ppm (*n* = 36)	7.80 (*P* < 0.05)			
		females, relative liver weight:		BMC_{05} = 144.7 ppm Adjusted BMC_{05} = 25.8 ppm	95% LCL_{05} = 76.3 ppm Adjusted 95% LCL_{05} = 13.6 ppm	F(1,156) = 0.34 *P*-value = 0.56
		control (*n* = 42)	5.59			
		25 ppm (*n* = 35)	5.71			
		100 ppm (*n* = 36)	5.99			
		400 ppm (*n* = 47)	6.35 (*P* < 0.05)			

[a] Adjusted from intermittent exposure (h/day, days/week) to continuous exposure.
[b] LCL = Lower confidence limit.

Table 3: Effect levels and benchmark doses for DMF, oral exposure.

		Data for calculating benchmark dose		Benchmark dose		
Study (reference)	**Effect level**	**Dose (mg/kg body weight per day)**	**Response**	**Parameter estimates**		**Goodness of fit**
Medium-term exposure						
Rat, Wistar 25 males and 25 females per group Dietary administration for 15 weeks (Becci et al., 1983)	LOEL = 69 mg/kg body weight per day, based upon a significant increase in relative liver weight in females at the two highest doses (NOEL = 20 mg/kg body weight per day)	males, relative liver weight: control (*n* = 25) 18 (*n* = 23) 61 (*n* = 25) 210 (*n* = 23)	 4.30 ± 0.09 4.51 ± 0.11 4.59 ± 0.08 4.99 ± 0.10 (*P* < 0.05)	BMD_{05} = 23.1 mg/kg body weight per day	95% LCL_{05} = 12.7 mg/kg body weight per day	F(1,92) = 0.73 *P*-value = 0.39
		females, relative liver weight: control (*n* = 25) 20 (*n* = 25) 69 (*n* = 24) 235 (*n* = 24)	 86 ± 0.06 89 ± 0.08 24 ± 0.12 (*P* < 0.05) 00 ± 0.12 (*P* < 0.05)	BMD_{05} = 35.9 mg/kg body weight per day	95% LCL_{05} = 15.7 mg/kg body weight per day	F(1,94) = 0.13 *P*-value = 0.72
Mouse, CD-1 30 males and 30 females per group dietary administration for 17 weeks (Becci et al., 1983)	LOEL = 96 mg/kg body weight per day, based upon statistically significant increase in relative liver weight in females (NOEL = 28 mg/kg body weight per day)	males, relative liver weight: control (*n* = 30) 22 (*n* = 28) 70 (*n* = 29) 246 (*n* = 29)	 5.3 ± 0.1 5.6 ± 0.1 5.8 ± 0.1 6.6 ± 0.1 (*P* < 0.01)	BMD_{05} = 21.3 mg/kg body weight per day	95% LCL_{05} = 7.6 mg/kg body weight per day	F(1,112) = 1.17 *P*-value = 0.28
		females, relative liver weight: control (*n* = 30) 28 (*n* = 29) 96 (*n* = 29) 326 (*n* = 30)	 5.1 ± 0.2 5.5 ± 0.1 5.9 ± 0.1 (*P* < 0.01) 6.6 ± 0.3 (*P* < 0.01)	BMD_{05} = 36.8 mg/kg body weight per day	95% LCL_{05} = 21.3 mg/kg body weight per day	F(1,114) = 0.14 *P*-value = 0.71

liver of rats exposed to DMF vapour for 18 weeks at a concentration of just 7.3 ppm (21.9 mg/m^3) (no further details provided in the citation) (Cai & Huang, 1979). Myocardial changes occurred in rabbits exposed to 40 ppm (120 mg/m^3) for 50 days (Arena et al., 1982).

8.4.2 Oral

In a 90-day dietary study, Crl:CD rats were exposed to 0, 10, 50, or 250 mg/kg body weight per day (Haskell Laboratory, 1960; Kennedy & Sherman, 1986). Mild effects on the liver (enlargement of hepatic cells) and haematological effects (anaemia, leukocytosis) were observed at 50 mg/kg body weight per day; at the top dose of 250 mg/kg body weight per day, weight gain was reduced, and the animals had slight anaemia, leukocytosis, and liver cell enlargement. Although there was an apparent increase in serum cholesterol in both sexes at the highest dose, statistical analyses were not presented. The no-observed-effect level (NOEL) was 10 mg/kg body weight per day. The lowest-observed-effect level (LOEL) is 50 mg/kg body weight per day, based upon a significant increase in relative liver weight in males.

In a second study involving larger group sizes, a different strain (Wistar), and more comprehensive tissue examination, growth was inhibited but no tissue lesions were observed in rats administered DMF in the diet for 15 weeks (Becci et al., 1983). Males received 0, 18, 61, or 210 mg/kg body weight per day, and females received 0, 20, 69, or 235 mg/kg body weight per day. The LOEL is 69 mg/kg body weight per day, based upon a significant increase in relative liver weight in females at the two highest doses (NOEL = 20 mg/kg body weight per day).

In the corresponding study in CD-1 mice involving dietary administration (males: 0, 22, 70, or 246 mg/kg body weight per day; females: 0, 28, 96, or 326 mg/kg body weight per day) for 17 weeks, there were no overt signs of toxicity and no notable effects on blood morphology, blood biochemistry, or urinary parameters (Becci et al., 1983). Microscopic examination of an extensive range of organ tissues revealed only mild effects on the liver in the majority of high-dose males and females. There was a dose-related increase in relative liver weight at all dose levels, although this was statistically significant only in the mid- and high-dose females and in the high-dose males. On this basis, the LOEL is 96 mg/kg body weight per day, based upon a significant increase in relative liver weight in females (NOEL = 28 mg/kg body weight per day).

In a submission to the US EPA Office of Toxic Substances, BASF (1984) reported that there were no adverse effects observed in beagle dogs (four males and four females per group) administered 0, 1.4, 7.0, or 34.8 mg/kg body weight per day (NOEL) in the diet for 13 weeks. The protocol included measurement of food consumption, measurement of body weight gain, hearing tests, ophthalmoscopic examination, clinical laboratory investigations, measurement of organ weights, and histopathological observations.

8.5 Long-term exposure and carcinogenicity

Information on the incidences of lesions in critical long-term studies is presented in Tables 2 and 3.

8.5.1 Inhalation

Malley et al. (1994) exposed Crl:CD BR rats for 6 h/day, 5 days/week, to 0, 25, 100, or 400 ppm (0, 75, 300, or 1200 mg/m^3) DMF vapour for 24 months. There were no overt signs of toxicity other than a reduction in weight gain in the rats exposed at 400 ppm (1200 mg/m^3) and, to a lesser extent and towards the end of the study, in males exposed at 100 ppm (300 mg/m^3). Haematological findings were normal, as were urinary analyses. There was a concentration-related increase in serum sorbitol dehydrogenase activity (indicative of hepatic effects) in the male and female rats at 100 and 400 ppm (300 and 1200 mg/m^3). Relative liver weights were increased in both sexes at 400 ppm (1200 mg/m^3), and microscopic examination revealed hepatic lesions (centrilobular hepatocellular hypertrophy, lipofuscin/haemosiderin accumulation, clear cell foci, and single-cell necrosis in males and high-dose females and focal cystic degeneration in males) at 100 and 400 ppm (300 and 1200 mg/m^3). Microscopic examination of an extensive range of tissues from the high-dose animals (and of selected tissues from the lower dose groups) revealed no other treatment-related lesions except in females, in which there was an increased incidence of uterine endometrial stromal polyps (1.7%, 5.1%, 3.4%, and 14.8% for control, low-, mid-, and high-dose females, respectively). Historical control data from the same laboratory indicated a highly variable incidence of endometrial stromal polyps (2–15% for 14 control groups, average 6.6%). The investigators concluded that DMF was not carcinogenic to rats under the conditions of exposure. The LOEC was 100 ppm (300 mg/m^3) (NOEC = 25 ppm [75 mg/m^3]), based upon a significant increase in centrilobular hepatocellular hypertrophy (both sexes), significant increase in hepatic accumulation of lipofuscin/haemosiderin (both sexes), and hepatic single-cell necrosis (females only).

Mice [Crl:CD 1 (ICR)BR] were exposed to 0, 25, 100, or 400 ppm (0, 75, 300, or 1200 mg/m^3) DMF for 6 h/day, 5 days/week, for 18 months (Malley et al., 1994). Haematological observations were normal. Relative liver weight was significantly increased at the two highest concentrations in males. Microscopic alterations in liver were observed at all levels of exposure. The authors concluded that DMF was not carcinogenic to mice under the conditions of the bioassay. The LOEC is 25 ppm (75 mg/m^3), based upon centrilobular hepatocellular hypertrophy (males), hepatic single-cell necrosis (males and females), and hepatic Kupffer cell hyperplasia/pigment accumulation (males).

8.5.2 Oral

An inadequate carcinogenicity study involving the administration of DMF in the drinking-water of BD rats at approximately 10 or 20 mg/kg body weight per day for 500 or 250 days, respectively, provided no evidence of tumour formation, although the extent of tissue examination was not specified (Druckrey et al., 1967). In female Mongolian gerbils administered DMF in the drinking-water at concentrations of 1.0–6.6% (around 5–40 mg/kg body weight per day) for up to 200 days, there were many early deaths at concentrations of 1.7% (around 7–11 mg/kg body weight per day) and above, and all DMF-exposed groups had liver degeneration and kidney congestion (Llewellyn et al., 1974).

8.5.3 Injection

In a study in hamsters investigating the carcinogenic activity of aflatoxins, there was no mention of any tumours in the DMF-treated controls. These animals (five males and five females) received weekly intraperitoneal injections of 0.1 ml of a 50% DMF solution (equivalent to approximately 47 mg DMF/kg body weight per injection) for 6–8.5 months and were then maintained untreated until they died (average life span 19 months) (Herrold, 1969). Although there were no increases in tumours following repeated intraperitoneal injections of DMF to rats for 10 weeks in a study reported in a secondary source, available information was inadequate to permit critical review (Kommineni, 1973).

8.6 Genotoxicity and related end-points

The following discussion is limited to results of assays for gene mutation and cytogenesis, i.e., those assays in which the end-points are most relevant to the assessment of DMF with respect to human health.

The results of assays for gene mutation *in vitro* were almost entirely negative. Of 20 identified assays in *Salmonella*, results were negative in 18 (Green & Savage, 1978; Purchase et al., 1978; Baker & Bonin, 1981; Brooks & Dean, 1981; Garner et al., 1981; Gatehouse, 1981; Ichinotsubo et al., 1981; MacDonald, 1981; Martire et al., 1981; Nagao & Takahashi, 1981; Richold & Jones, 1981; Rowland & Severn, 1981; Simmon & Shepherd, 1981; Skopek et al., 1981; Venitt & Crofton-Sleigh, 1981; Antoine et al., 1983; Falck et al., 1985; Mortelmans et al., 1986), and two had equivocal results (Hubbard et al., 1981; Trueman, 1981). Results in six assays in *Escherichia coli* were all negative (Gatehouse, 1981; Matsushima et al., 1981; Mohn et al., 1981; Thomson, 1981; Venitt & Crofton-Sleigh, 1981; Falck et al., 1985).

Although fewer assays for cytogenetic effects and genotoxicity *in vitro* were identified than for gene mutation, results were also predominantly negative. In assays for chromosomal aberrations (CAs), results were negative for human lymphocytes (Antoine et al., 1983) and Chinese hamster ovary (CHO) (Natarajan & van Kesteren-van Leeuwen, 1981) and weakly positive in human peripheral lymphocytes (Koudela & Spazier, 1979). In three mouse lymphoma assays, results were negative (Jotz & Mitchell, 1981; Mitchell et al., 1988; Myhr & Caspary, 1988) and one was weakly positive (McGregor et al., 1988). Results of *in vitro* tests for sister chromatid exchange (SCE) were negative in three assays in CHO (Evans & Mitchell, 1981; Natarajan & van Kesteren-van Leeuwen, 1981; Perry & Thomson, 1981) and one in human lymphocytes (Antoine et al., 1983). Assays for unscheduled DNA synthesis (UDS) were negative in human fibroblasts (Agrelo & Amos, 1981; Robinson & Mitchell, 1981), mouse hepatocytes (Klaunig et al., 1984), and HeLa cells (Martin & McDermid, 1981), while in assays in rat hepatocytes, results were both negative (Ito, 1982) and positive (Williams, 1977). Results of assays for DNA repair in mouse (McQueen et al., 1983) and hamster (McQueen et al., 1983) hepatocytes were also negative. An assay for DNA repair in human hepatocytes had negative results (McQueen et al., 1988).

The database for genotoxicity studies *in vivo* is more limited than that for *in vitro* studies.

In two adequate assays for micronucleus induction, results were negative (Kirkhart, 1981; Antoine et al., 1983). In the latter study, dose levels were too widely spaced, and the top dose was 2000 mg/kg body weight. Results were also negative in two assays in which there were no positive controls (Salamone et al., 1981; Tsuchimoto & Matter, 1981). It should be noted that Salamone et al. (1981) observed no effect at doses up to 80% of the LD_{50}. An assay in which an increase in

micronuclei was observed in bone marrow of mice was reported only as an abstract (Ye, 1987), although a dose–response was not clear. Although six dose levels were included in the protocol, the highest dose was only 20 mg/kg body weight (oral LD_{50} values in laboratory animals range from 2000 to 7000 mg/kg body weight).

Negative results were reported in assays for chromosomal damage in bone marrow of rats (Sheveleva et al., 1979; McGregor, 1981) and dominant lethal assays in rats (Lewis et al., 1979; McGregor, 1981; Cragin et al., 1990). Limited reporting (abstracts, secondary sources) precluded critical review of these studies.

Quantitative data were not presented in a report of an assay in which SCEs were not observed in bone marrow of mice (Paika et al., 1981).

8.7 Reproductive toxicity

8.7.1 Effects on fertility

Effects on organ weights or histopathological effects in the reproductive organs have not been observed in medium-term or long-term studies in rats or mice following inhalation or oral exposure (Becci et al., 1983; Craig et al., 1984; Kennedy & Sherman, 1986; NTP, 1992a; Malley et al., 1994). In several of these bioassays, additional reproductive end-points were examined. These included sperm density, motility, or count and length of diestrus in rats and mice exposed for 13 weeks to concentrations up to 800 ppm (2400 mg/m^3) (NTP, 1992a) and semen volume and sperm motility, morphology, or count in a limited number of monkeys exposed to 500 ppm (1500 mg/m^3) (Hurtt et al., 1992). In none of these investigations, however, were there adverse effects on reproductive parameters at concentrations or doses less than those at which hepatic effects were observed; indeed, the only effect reported was prolonged diestrus in female rats exposed to 800 ppm (2400 mg/m^3) for 13 weeks (NTP, 1992a).

Few studies were identified in which the protocols were designed specifically to address reproductive toxicity. In a study reported as abstracts (Lewis et al., 1979; Cragin et al., 1990), exposure of male Sprague-Dawley rats to 30 or 300 ppm (90 or 900 mg/m^3) for 6 h/day for 5 days did not result in histopathological changes in reproductive organs after 6 weeks. Pairing of the exposed males with unexposed females for 6 weeks after exposure resulted in a reduced number of viable fetuses per dam in the low-dose group only.

In a multi-generation study in Swiss mice, DMF was administered in the drinking-water at concentrations of 0, 1000, 4000, or 7000 mg/litre (NTP, 1992b; Fail et al., 1998). Litters from F0 animals were sacrificed immediately. At week 16, pairs were separated and the final litters reared to postnatal day 21, then entered into an F1 fertility assessment. A crossover mating trial was also carried out with the F0 mice. The lowest level of exposure (1000 mg/litre; average 219 mg/kg body weight per day) was designated by the authors as the maximum tolerated dose (LOEL) for the F0 mice, based upon increased relative liver weight in males and females and increased relative kidney and adrenal weight in females. Reproductive effects in F0 mice included reduced fertility and fecundity at 4000 and 7000 mg/litre. The crossover trial identified females as the affected sex. Following F1 mating, both F2 litter size and live pup weight were reduced at all doses. At necropsy, the body weight of F1 males and females was reduced at the two highest doses, and both absolute and relative liver weights were increased at all doses. The authors concluded that both reproductive and developmental toxicity occurred at the two highest doses (4000 and 7000 mg/litre) in the F0 mice and at all dose levels ($1000 mg/litre) in the F1 mice.

No abnormalities were observed in sperm in an adequate single-injection study in mice, for which few details were presented (Antoine et al., 1983). Although negative results were reported in other assays in mice, quantitative data were not presented (Topham, 1980, 1981) or only a secondary source was available (McGregor, 1981).

8.7.2 Developmental toxicity

The database on developmental toxicity is more extensive, with numerous studies having been conducted in various species by the inhalation, oral, and dermal routes. Emphasis here is on the most recent studies for which protocols and reporting are most extensive.

In studies in which DMF has been administered by inhalation or ingestion, it has been, at most, weakly teratogenic, with malformations being observed only at high doses that were maternally toxic (450 ppm [1350 mg/m^3] by inhalation in rabbits; 503 mg/kg body weight per day following ingestion in rats), based on consideration of maternal body weight and signs of overt toxicity (Hellwig et al., 1991). In general, DMF has induced primarily fetotoxic effects most often at maternally toxic concentrations or doses (100 mg/kg body weight per day by stomach tube in rats) (Saillenfait et al., 1997) but occasionally in the absence of maternal toxicity, based on determination of body weight gain and overt signs. For example, Lewis et al. (1992) reported maternal weight gain in Crl:CD rats at 300 ppm (900

mg/m^3) (maternal LOEC), but not at 30 ppm (90 mg/m^3), at which concentration there was a slight but significant reduction in fetal weight. The mean fetal weights of control, low-dose, and high-dose groups were 5.5 ± 0.2, 5.5 ± 0.4, and 5.3 ± 0.2 g, respectively ($P < 0.05$ for both low- and high-dose groups).

The pattern of results of studies by the dermal route was similar, with malformations being observed in rats only at doses that were maternally toxic based on examination of weight gain and overt signs of toxicity only (944 mg/kg body weight per day in rats; 400 mg/kg body weight per day in rabbits; 944 mg/kg body weight per day in mice) (Hellwig et al., 1991). In one of the relatively recent investigations by other authors (Hansen & Meyer, 1990), fetotoxic effects (delayed ossification) only were observed at doses (945 mg/kg body weight per day) at which there were no effects on maternal weight gain and no overt signs of maternal toxicity.

Klug et al. (1998) carried out a mouse limb bud assay with DMF, HMMF, NMF, SMG (a synthesis product of glutathione and methyl isocyanate), *S*-(*N*-methyl-carbamoyl)cysteine (SMC), *N*-acetoximethyl-*N*-methylformamide (AMMF), AMCC, L-cysteine, and glutathione. There were no signs of adverse developmental effects caused by DMF, NMF, HMMF, AMMF, L-cysteine, or glutathione. However, a pronounced impact upon growth and development was observed for AMCC, SMC, and SMG (metabolites resulting from the glutathione binding pathway). The authors concluded that the developmental toxicity of DMF in different species is related to the magnitude of glutathione binding.

8.8 Neurological effects

In male Wistar rats exposed to 0, 7, 35, or 65 mg DMF/kg body weight per day in drinking-water for either 2 or 7 weeks, glial cell fractions were isolated from the left cerebral hemisphere and assayed for activity of acid proteinase and 2',3'-cyclic nucleotide 3'-phosphohydrolase (Savolainen, 1981). The right cerebral hemisphere was assayed for RNA, glutathione, and activities of succinate dehydrogenase and azoreductase. After 2 weeks, there was a dose-related increase in activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase, which was significant ($P < 0.001$) at all levels of exposure. After 7 weeks of exposure to 0, 8, 39, or 75 mg/kg body weight per day, the intake of drinking-water was significantly reduced at all levels of exposure. There was also a significant reduction in activity of azoreductase and succinate dehydrogenase (uneven dose–response).

9. EFFECTS ON HUMANS

Consistent with the results of studies in experimental animals, available data from case reports and cross-sectional studies in occupationally exposed populations consistently indicate that the liver is the target organ for the toxicity of DMF in humans. The profile of effects is consistent with that observed in experimental animals, with related symptoms, increases in serum hepatic enzymes, and histopathological effects being reported.

9.1 Effects on the liver

Case reports in workers acutely exposed to DMF confirm that the liver is the target organ, with hepatic effects and associated disorders of the digestive system being reported. Symptoms include abdominal pain, anorexia, incoordination, and jaundice, as well as nausea, vomiting, and diarrhoea; nasal and skin irritation have also been reported (Tolot et al., 1968; Potter, 1973; Chary, 1974; Chivers, 1978; Guirguis, 1981; Paoletti et al., 1982a, 1982b; Riachi et al., 1993; Drouet D'Aubigny et al., 1998; Huang et al., 1998). Changes in both liver function (Weiss, 1971; Potter, 1973; Guirguis, 1981; Paoletti et al., 1982b; Riachi et al., 1993; Drouet D'Aubigny et al., 1998) and morphology (Tolot et al., 1968; Riachi et al., 1993) have also been observed. In one of the few reports where there was some indication of magnitude of exposure, hepatic impairment (marked increases in serum levels of ALT, aspartate aminotransferase [AST], AP, and bilirubin, together with fulminant hepatitis and jaundice) was reported in a woman who ingested about 0.6 g DMF/kg body weight (in a formulation containing other ingredients) in a suicide attempt (Nicolas et al., 1990). Similarly, clinical measurements were carried out in a patient who intravenously injected (presumably) 50 ml of a veterinary euthanasia drug containing DMF as a solvent (Buylaert et al., 1996). Serum AST and ALT increased, there was a transient rise in total serum bilirubin, and prothrombin time decreased. AP levels remained within the normal range.

Alcohol intolerance, characterized by flushing of the face, dizziness, nausea, and tightness of the chest, has been widely reported among DMF-exposed workers (Lyle, 1979; Lyle et al., 1979; Lauwerys et al., 1980; Yonemoto & Suzuki, 1980; Paoletti & Iannaccone, 1982; Paoletti et al., 1982a; Tomasini et al., 1983; Cirla et al., 1984; Redlich et al., 1988, 1990; Wang et al., 1989, 1991; Cai et al., 1992; Fiorita et al., 1997; Wrbitzky, 1999). While it is difficult to establish with

Table 4: Effects of DMF exposure on hepatic function in humans.[a]

Concentration[b]	Effect on liver enzymes	Exposed population	Confounders	Reference
<10–60 ppm; random area sampling	increase	183 workers	some workers were also exposed to solvents	Wang et al. (1989, 1991)
10–42 ppm; area monitoring	increase	13 workers	few details reported	Yang et al. (1994)
1–27 ppm	no effect	27 workers		Paoletti & Iannaconne (1982)
5–20 ppm	increase (significance not reported)	13 workers	exposure to solvents	Tomasini et al. (1983)
3–20 ppm (TWA, 7 ppm); personal sampling	significant increase	100 workers		Cirla et al. (1984)
0.3–15.5 ppm (usually <10 ppm); static area sampling	no effect	22 workers		Lauwerys et al. (1980)
1–5 ppm; personal and area sampling	no effect	6 workers		Yonemoto & Suzuki (1980)
4–8 ppm (mean 6 ppm); sampling not specified	no effect	28 workers		Catenacci et al. (1984)
0.2–8 ppm; area sampling	increase (significance not reported)	26 workers	concomitant exposure to acrylonitrile	Major et al. (1998)
7 ppm; area sampling at different workplaces	significant increase	75 workers		Fiorito et al. (1997)
0.1–7 ppm; personal sampling	no effect	207 workers	some workers were also exposed to toluene	Cai et al. (1992)
up to 2.3 ppm; personal sampling	no effect	126 workers		Wrbitzky & Angerer (1998); Wrbitzky (1999)

[a] See text for more detailed descriptions of highlighted studies.
[b] 1 ppm = 3 mg/m^3.

any certainty a lowest concentration at which increases in these subjective symptoms first appear, they have been associated with mean or median levels of 10 ppm (30 mg/m^3) (Lauwerys et al., 1980; Yonemoto & Suzuki, 1980; Cai et al., 1992; Fiorito et al., 1997); in a recent study, some workers reported symptoms upon exposure to concentrations for which the median value was as low as 1.2 ppm (3.6 mg/m^3) (Wrbitzky, 1999).

Levels of serum hepatic enzymes in populations occupationally exposed to DMF have been determined in several cross-sectional studies. A brief overview of the information on exposure–response derived from these studies is summarized in Table 4.

While there have been considerable variations in the size of study populations, magnitude and duration of exposure, extent of exposure to other substances, and adequacy of reporting in these investigations, there is a consistent pattern of increase in serum enzymes in workers with relatively higher exposures in these investigations, some of which included individual monitoring. In summary, the results concerning exposure–response are consistent across studies, with increases in serum hepatic enzymes not being observed at concentrations in the range of 1–6 ppm (3–18 mg/m^3). At higher levels of exposure (>7 ppm [>21 mg/m^3]), increased serum levels of hepatic enzymes have been observed consistently.

There were three studies identified (highlighted in Table 4) for which TWA exposures were presented and which can serve, therefore, as the basis for at least crude estimates of exposure–response. These are described in more detail here. It should be noted, though, that the monitored levels in these studies do not take into account potential additional dermal exposure.

In a carefully conducted investigation of liver function in 75 workers in a synthetic leather factory, geometric mean levels of DMF in the air based on 8-h area sampling in various working locations were approximately 20 mg/m^3 (~7 ppm) (range 2–40 mg/m^3) (Fiorito et al., 1997). It was reported that the study subjects worked in a factory that produces synthetic leather using polyurethane resin, pigments, and large amounts of DMF (about 14 tonnes/day), where skin contact with liquid DMF was also possible. The mean duration of employment was 3.8 years. The control group consisted of 75 unexposed workers similar in age, sex, social status, and residence. Confounding by

alcohol consumption and pre-existing liver disease was minimized through selection criteria for study subjects. Analysis of paired enzymes was also conducted. All workers underwent a complete physical examination, with liver function tests for serum AST, ALT, (-glutamyl transpeptidase ((-GT), AP, bile acids (BA), bilirubin, serum cholesterol and triglycerides, and markers for hepatitis A, B, and C. Gastrointestinal symptoms (stomach pain, nausea, appetite loss) were reported by 50% of the DMF-exposed workers, and 40% had symptoms such as face flushing, palpitation, headache, dizziness, or tremors following alcohol consumption. (Many avoided alcohol as a result.) Mean serum ALT (28.8 vs. 21.9 IU/litre), AST (26.5 vs. 21.1 IU/litre), (-GT (29.5 vs. 14.2 IU/litre), and AP (75.7 vs. 60.8 IU/litre) were significantly higher in 12 of 75 workers in the exposed group ($P < 0.001$); 17/75 (23%) had abnormal liver function, compared with only 4% of controls. Multivariate analyses confirmed that ALT, AST, and (-GT were significantly correlated with cumulative DMF exposure. The analyses controlled for factors such as body mass index, alcohol intake, serum cholesterol, and hepatitis markers, which did not explain the observed effects.

Catenacci et al. (1984) investigated liver function (serum glutamate–oxalate transaminase [SGOT], serum glutamate–pyruvate transaminase [SGPT], (-GT, and AP) in workers employed for at least 5 years in an acrylic fibre plant; no mention was made of exposure to other solvents. The first group of 28 subjects worked in the spinning department, where DMF exposure (8-h TWA) ranged from 12 to 25 mg/m^3, with a mean of 18 mg/m^3 (4–8 ppm, mean 6 ppm). The second group consisted of 26 subjects exposed, in the polymer department, to DMF at (8-h TWA) 1.8–5 mg/m^3, with a mean of 3 mg/m^3 (0.6–1.8 ppm, mean 1 ppm). A control group consisted of 54 subjects matched for age, smoking/alcohol consumption, and history of liver disease, who had never been occupationally exposed to solvents. The data on which the estimated TWA exposures were based were not reported. Mean serum values for SGOT (20.74, 21.06, and 20.17 mU/ml for 6 ppm, 1 ppm, and control groups, respectively), SGPT (19.76, 21.26, and 26.09 mU/ml for 6 ppm, 1 ppm, and control groups, respectively), (-GT (36.37, 28.34, and 40.76 mU/ml for 6 ppm, 1 ppm, and control groups, respectively), and AP (154.42, 150.35, and 153.07 mU/ml for 6 ppm, 1 ppm, and control groups, respectively) did not differ among the three groups and were within the normal ranges. Few additional details were presented in the published account of this study.

Cirla et al. (1984) carried out a clinical evaluation of 100 workers in synthetic polyurethane leather production exposed to a mean TWA concentration (determined by personal sampling) of 22 mg/m^3 (range 8–58 mg/m^3) (mean TWA 7 ppm; range 3–19 ppm). The mean exposure period was 5 years (range 1–15 years). The workers were also exposed to small (but unspecified) quantities of toluene, methyl ethyl ketone (MEK), ethyl acetate, and isopropyl and isobutyl alcohol. Study subjects were selected to minimize large variations in exposure; those with histories of possible accidental exposures were also excluded. The referent group was 100 workers at the same or similar factories, without exposure to any solvents or toxic metals, matched by sex, age group, alcohol history, smoking habits, coffee intake, socio-economic status, residence, and dietary customs. Clinical evaluation was carried out and a laboratory assessment was performed for blood cell counts and serum AP, AST, ALT, and (-GT. Serum (-GT was abnormally high in 25/100 exposed and only 10/100 referents ($P < 0.01$). Higher prevalences in the exposed group for abnormally high serum levels of AST (9 vs. 3) and ALT (12 vs. 8) were not statistically significant. AP values were normal in all subjects. When subjects who had not modified their alcohol consumption upon working with DMF were considered, the effect was still evident. Several symptoms, including headache, dyspepsia, and digestive impairment, characteristic of effects on the liver were also associated with exposure to DMF.

Histopathological changes in liver have also been reported in occupationally exposed workers, although quantitative data on levels of exposure are not well documented. Tomasini et al. (1983) reported hepatic pain and palpable liver in 4 of 13 workers exposed to 5–20 ppm (15–60 mg/m^3) DMF (and other solvents), ranging from a few weeks to 4 years. Redlich et al. (1990) carried out biopsies of liver from workers heavily exposed to DMF (and other solvents; quantitative data not reported). Workers exposed for less than 3 months had hepatocellular necrosis, enlarged Kupffer cells, microvesicular steatosis, complex lysosomes, and pleomorphic mitochondria. The livers of workers exposed for longer terms (14–120 months) had fatty changes with occasional lipogranuloma.

9.2 Cardiac effects

Excess mortality from ischaemic heart disease in DMF-exposed workers in a US acrylonitrile fibre plant was observed in a historical cohort study (Chen et al., 1988b). Between 1950 and 1982, there were 62 deaths due to ischaemic heart disease (40.3 expected from company rates; $P < 0.01$). The increase was not significant in comparison with the state (South Carolina) rates. A similar observation was made for a second group of 1329 employees at the plant who were potentially exposed to

both DMF and acrylonitrile (65 deaths observed, 48.3 expected from company rates; $P < 0.05$). However, the observed number of deaths was not significantly higher than that which would be expected from either state or national rates, possibly due to a "healthy worker effect." Lifestyle factors such as alcohol and tobacco consumption were suggested to be more likely causes than exposure to DMF, although the specific basis for this contention was not specified (Chen et al., 1988b). The authors noted that South Carolina has a higher ischaemic heart disease mortality rate than the USA.

No convincing evidence of adverse effects on cardiac function was seen in a limited study in which electrocardiographic (ECG) monitoring was carried out on workers at a small synthetic leather plant where DMF was used. Monitoring of eight workers over a workshift revealed possible mild effects (isolated ventricular premature beats after 2 h of work, without "pathological alteration" of the ECG) in one worker (Taccola et al., 1981). In a brief report, ECG changes in workers exposed to DMF were reported (<3 ppm [<9 mg/m^3], with peaks up to 1500 ppm [4500 mg/m^3], plus skin exposure), but little detail was provided (Kang-De & Hui-Lan, 1981).

Cardiac disturbances, including tachycardia and palpitations, have occasionally been observed in cross-sectional studies of DMF-exposed workers (Lyle, 1979; Lyle et al., 1979; Kang-De & Hui-Lan, 1981; Cirla et al., 1984; Fiorito et al., 1997). Sometimes, the palpitations followed alcohol ingestion (Lyle, 1979; Lyle et al., 1979; Fiorito et al., 1997).

9.3 Cancer

Data on the incidence or mortality of cancer associated with exposure to DMF are limited to case reports of testicular tumours and single well conducted and reported cohort and case–control studies of occupationally exposed populations (Chen et al., 1988a; Walrath et al., 1989). In the cohort study of 3859 actively employed workers with potential exposure to DMF and to DMF and acrylonitrile in an acrylonitrile fibre production facility, the incidences of cancer of the buccal cavity/pharynx, lung, prostate, stomach, nervous system, and bladder were considered in relation to level of and, for some tumours, duration of exposure and were compared with company and national rates. Level of exposure was classified as low (approximately <10 ppm (<30 mg/m^3)), moderate (sometimes above 10 ppm [30 mg/m^3]), or high, although quantitative data were not reported (Chen et al., 1988a). In an additional case–control study, cancers of the buccal cavity/pharynx (n = 39), liver (n = 6), prostate (n = 43), and testis (n = 11) and malignant melanoma of the skin (n = 39) were examined in approximately 8700 workers from four plants, which included a DMF production plant, two acrylic fibre plants that used DMF as a spinning solvent, and a plant using the chemical as a solvent for inks (Walrath et al., 1989).

Three cases of testicular germ cell tumours that occurred during 1981–1983 among 153 white men who repaired the exterior surfaces and electrical components of F4 Phantom jets in the USA were reported by Ducatman et al. (1986), which led to surveys of two other repair shops at different locations, one in which F4 Phantom jets were repaired and one where other types of aircraft were repaired. Four of 680 workers in the F4 Phantom shop had testicular germ cell cancers (approximately one expected) diagnosed during 1970–1983. No cases were reported in the other facility. All seven men had long histories in aircraft repair; although there were many common exposures to solvents in the three facilities, the only one identified as unique to the F4 Phantom jet aircraft repair facilities was to a solvent mixture containing 80% DMF (20% unspecified). Three of the cases had been exposed to this mixture with certainty, and three had probably been exposed. Of the seven cases, five were seminomas and two were embryonal cell carcinomas.

Levin et al. (1987) and Frumin et al. (1989) reported three cases of embryonal cell carcinoma of the testis in workers at one leather tannery in the USA, where it was reported that DMF as well as a wide range of dyes and solvents were used, including such testicular toxins as 2-ethoxyethanol and 2-ethoxyethanol acetate. The latency period ranged from 8 to 14 years. No additional cancers were reported in a screening effort undertaken to identify additional testicular cancers in 51 of the 83 workers at the leather tannery where the three cases were reported (Calvert et al., 1990).

In an investigation of cancer incidence at a plant producing acrylonitrile fibres, compared with company and national rates, there was no increase in the incidence of testicular cancer in 2530 actively employed workers exposed to DMF only. When the data from this cohort were grouped with data from 1329 workers exposed to both DMF and acrylonitrile, there was only one case of testicular cancer, versus 1.7 expected (confidence interval [CI] not reported) (Chen et al., 1988a).

There was no increase in cancer of testis (odds ratio = 0.91; 95% CI = 0.1–8.6; observed number of cases = 11) in the case–control study described above in which the cases were drawn from a population of

approximately 8700 workers involved in production or use of DMF at four plants (Walrath et al., 1989, 1990). For each case, two controls were selected, matched for age, sex, payroll class, and plant. Potential exposure to DMF was classified as low or moderate based on job title/work area combinations and monitoring data.

Chen et al. (1988a) observed a significant increase in prostate cancer (10 observed vs. 5.1 expected from company rates and 5.2 expected from national rates; $P < 0.10$ for both comparisons) in the 3859 workers exposed either to DMF or to both DMF and acrylonitrile. However, when only DMF-exposed workers (2530) were considered, the standardized incidence rate (SIR) (4 observed vs. 2.4 expected from company rates) was not significant. The odds ratio for prostate cancer in the case–control study of the 8700 DMF-exposed workers from four plants was not significantly elevated (1.48; 95% CI = 0.59–3.74; 43 cases) (Walrath et al., 1989, 1990). When analyses were carried out separately for each of the four plants, an increased incidence was observed only at one plant, where the exposure to DMF was lower and the number of cases was fewer than at the other plants. Adjustment for assumed latency period did not alter the odds ratio. There was no relationship with duration of exposure.

Chen et al. (1988a) also reported a significant increase of cancer of the buccal cavity/pharynx (9 observed vs. 1.6 expected from company rates; $P < 0.10$) in the 2530 DMF-exposed workers (confidence intervals not reported). When combined with data from 1329 workers exposed to both DMF and acrylonitrile, the increase (11 observed) was significant when compared with the company rate (3.2 expected; $P < 0.01$), but not compared with the national rate (6.6 expected). There was no relation to either level or duration of exposure. All cases were heavy, long-term smokers. There was no increase in risk of cancer of the buccal cavity/pharynx in the case–control study of workers at the four plants mentioned above (odds ratio = 0.89; 90% CI = 0.35–2.29; 39 cases) (Walrath et al., 1989, 1990).

9.4 Genotoxicity

Seven studies were identified in which the genotoxicity of DMF in humans has been examined. Four of these studies were critically reviewed by IARC (1999) and were described therein as follows.

Berger et al. (1985) reported that the prevalence of CAs was higher in the blood lymphocytes of 20 workers exposed to DMF, NMF, and dimethylamine than in 18 unexposed workers at the same factory (1.4% vs. 0.4%; statistical significance not provided). The mean concentrations 1 year prior to blood sampling were 12.3 mg/m^3 for DMF, 5.3 mg/m^3 for NMF, and 0.63 mg/m^3 for dimethylamine. However, the control group had an unusually low level of chromosome breaks. The IARC Working Group noted that the possible effect of smoking was not addressed.

A higher incidence of CAs was observed in the lymphocytes of about 40 workers exposed to DMF than in an unspecified control group (2.74–3.82% vs. 1.10–1.61%; $P < 0.05$). The range of exposure to DMF was 150–180 mg/m^3. Workers were also exposed to trace amounts of MEK, butyl acetate, toluene, cyclohexanone, and xylene. After technological improvements designed to reduce DMF exposure levels (range 35–50 mg/m^3), the frequency of aberrant cells decreased to 1.49–1.59% (Koudela & Spazier, 1981).

Although Sram et al. (1985) reported in an abstract that there was no evidence of increased frequency of CA in peripheral lymphocytes in workers exposed to DMF, no details were provided.

Seiji et al. (1992) reported that the mean SCE rate was higher in the blood cells of 22 women exposed to three concentrations of DMF (0.3–5.8 ppm [0.9–17.4 mg/m^3]) in a leather production factory than in 22 unexposed controls from the same factory, matched by sex, age, and residence. None of the women smoked tobacco or drank alcohol. The incidence of SCEs was significantly increased in a dose-related manner in the mid- and high-exposure groups.

Based on review of these studies, IARC (1999) concluded that "The positive data for cytogenetic damage in humans occupationally exposed to it are not very convincing."

Three relevant reports, including one for which only an abstract was identified in which few details were provided (Haber et al., 1990), were identified in addition to those reviewed by IARC (1999). The two investigations for which reporting was adequate are described here.

Major et al. (1998) reported that for workers with 3–10 years of occupational exposure to undefined levels of DMF and/or acrylonitrile, the prevalence of peripheral lymphocytes with CAs was increased compared with unexposed controls (see below). After a further 7 months of exposure (to DMF at 0.2–8 ppm [0.6–24 mg/m^3] and to acrylonitrile at 0–17.6 mg/m^3), the incidence in the exposed group increased to 5.1% but did not increase further up to 20 months. The incidence of SCEs was also

higher than control values at the start of the 20-month study and remained higher at 7 and 20 months. The UDS level was similar to that in controls when the study started, but had increased in the exposed group by month 7. In addition to concomitant exposure to acrylonitrile, current smoking was also a confounding factor, with CA and SCE yields being significantly higher in exposed smokers than in exposed non-smokers. Nevertheless, CA yields at 7 months were significantly higher in exposed non-smokers than in control non-smokers and in exposed smokers than in control smokers.

Cheng et al. (1999) measured SCE frequency in peripheral lymphocytes of workers at a resin synthesis plant. Nine workers had low exposure (median 5.2 ppm [15.6 mg/m^3]; range 0.9–5.3 ppm [2.7–15.9 mg/m^3]), and 20 workers had high exposure (median 24.8 ppm (74.4 mg/m^3); range 11.4–83.3 ppm [34.2–249.9 mg/m^3]). There were no differences between the two groups; there was no additional control population.

Results of studies on genotoxicity conducted since the IARC evaluation have not contributed materially to the database, which was considered by IARC (1999) not to provide convincing evidence. Certainly, the results, when taken as a whole, are inconsistent and not readily explained by variations in exposure.

10. EFFECTS ON OTHER ORGANISMS IN THE LABORATORY AND FIELD

DMF has been the focus of several toxicity studies conducted on a range of species. The most sensitive end-points for terrestrial and aquatic organisms are presented below and are summarized in Table 5.

10.1 Aquatic environment

A number of studies are available for a range of taxa, including protozoa, blue-green algae, diatoms, green algae, macrophytes, molluscs, oligochaetes, crustaceans, insect larvae, and fish.

For four species of fish, EC_{50} and LC_{50} values ranged from approximately 7100 to 12 000 mg/litre (Batchelder, 1976; Johnson & Finley, 1980; Call et al., 1983; Poirier et al., 1986; Groth et al., 1994). The most sensitive fish species appears to be the bluegill (*Lepomis macrochirus*), with an LC_{50} of 7100–7500 mg/litre.

Aquatic invertebrates tested include the water flea (*Daphnia magna*) and various species of insect larvae. The water flea appears to be the most sensitive invertebrate, with a NOEL of 1140 mg/litre. Acute end-points (EC_{50} and LC_{50}) for *Daphnia magna* range from 12 400 to 15 700 mg/litre, whereas chronic studies provide end-points for mortality between 1140 and 3721 mg/litre (Call et al., 1983; Leblanc & Surprenant, 1983; Adams & Heidolph, 1985; Poirier et al., 1986; Ziegenfuss et al., 1986; Sebaugh et al., 1991). The 48-h LC_{50}s obtained for various species of insect larvae were much higher and ranged from 33 500 to 36 200 mg/litre (Call et al., 1983; Poirier et al., 1986; Ziegenfuss et al., 1986).

The most sensitive alga appears to be *Selenastrum capricornutum*, with a 14-day NOEC value for growth inhibition of 480 mg/litre (Hughes & Vilkas, 1983). Results for two other green algae range from 8900 to 10 000 mg/litre (Stratton & Smith, 1988; El Jay, 1996). Peterson et al. (1997) obtained an IC_{25} for growth inhibition of 6200 mg/litre for the diatom *Nitzschia* sp. In the same study, blue-green algae appeared to be the least sensitive, with IC_{25}s for growth inhibition ranging from 7000 to 15 100 mg/litre for three tested species (Peterson et al., 1997), a finding that differs from earlier data (Stratton, 1987). Because of the high degree of quality assurance/quality control associated with the Peterson et al. (1997) study, these data are considered as definitive levels of toxicity to blue-green algae.

Rajini et al. (1989) measured the lethal response of the ciliated protozoan *Paramecium caudatum* to acute (10-min and 4-h) exposures to DMF. The 4-h LC_{50} was found to be 20 465 mg/litre. A recent paper reports EC_{50}s of 8190–9870 mg/litre for deformations and LC_{50}s of 19 700–31 700 mg/litre for the protozoan *Spirostomum ambiguum* (Nalecz-Jawecki & Sawicki, 1999).

Marine organisms tested include the bacteria *Vibrio fischeri*, the common shrimp (*Crangon crangon*), and a fish, the winter flounder (*Pleuronectes americanus*). For the decrease in luminescence in *Vibrio fischeri*, the 5-min EC_{50} value of 20 000 mg/litre (Curtis et al., 1982) is in the same order of magnitude as the values (13 260–14 830 mg/litre) obtained by Harwood[1] with a 15-min exposure. IC_{25} values calculated by Harwood[1] with the same data set range from 5830 to 6730 mg/litre.

[1] Personal communications from M. Harwood, Environment Canada, to A. Chevrier, Environment Canada, dated 2 and 5 December 1997.

Table 5: Toxicity of DMF to various organisms.

Test species	Latin name	End-point	Range	References
Bacteria	*Vibrio fischeri*	5-min EC_{50} light production	20 000 mg/litre	Curtis et al. (1982)
Bacteria	*Vibrio fischeri*	15-min IC_{50} light inhibition 15-min IC_{25} light inhibition	13 260–14 830 mg/litre 5830–6730 mg/litre	Harwood[a]
Protozoan	*Paramecium caudatum*	4-h LC_{50} mortality	20 465 mg/litre	Rajini et al. (1989)
Protozoan	*Spirostomum ambiguum*	24-h EC_{50} deformations 24-h LC_{50} mortality 48-h EC_{50} deformations 48-h LC_{50} mortality	9870 mg/litre 31 700 mg/litre 8190 mg/litre 19 700 mg/litre	Nalecz-Jawecki & Sawicki (1999)
Blue-green algae	*Nostoc* sp.	10- to 14-day EC_{50} growth inhibition test	<480 mg/litre	Stratton (1987)
Blue-green algae	*Anabaena* sp.	10- to 14-day EC_{50} growth inhibition test	<480 mg/litre	Stratton (1987)
Blue-green algae	*Anabaena cylindrica*	10- to 14-day EC_{50} growth inhibition test	<480 mg/litre	Stratton (1987)
Blue-green algae	*Anabaena variabilis*	10- to 14-day EC_{50} growth inhibition test	<480 mg/litre	Stratton (1987)
Blue-green algae	*Anabaena inaequalis*	10- to 14-day EC_{50} growth inhibition test	5700 mg/litre	Stratton (1987)
Blue-green algae	*Anabaena flos-aquae*	48-h IC_{25} growth inhibition	15 100 mg/litre	Peterson et al. (1997)
Blue-green algae	*Microcystis aeruginosa*	48-h IC_{25} growth inhibition	7000 mg/litre	Peterson et al. (1997)
Blue-green algae	*Oscillatoria* sp.	48-h IC_{25} growth inhibition	10 400 mg/litre	Peterson et al. (1997)
Diatom	*Nitzschia* sp.	48-h IC_{25} growth inhibition	6200 mg/litre	Peterson et al (1997)
Green algae	*Selenastrum capricornutum*	48-h IC_{25} growth inhibition	7700 mg/litre	Peterson et al. (1997)
Green algae	*Selenastrum capricornutum*	72-h IC_{25} growth as cell numbers	3420–6280 mg/litre	Harwood[a]
Green algae	*Selenastrum capricornutum*	growth at day 4	inhibition at 5000 mg/litre	El Jay (1996)
Green algae	*Selenastrum capricornutum*	growth inhibition NOEC	480 mg/litre	Hughes & Vilkas (1983)
Green algae	*Selenastrum capricornutum*	growth at day 4	stimulation at 1000 mg/litre	El Jay (1996)
Green algae	*Chlorella vulgaris*	growth at day 4	inhibition at 10 000 mg/litre	El Jay (1996)
Green algae	*Chlorella vulgaris*	growth at day 4	stimulation at 1000 mg/litre	El Jay (1996)
Green algae	*Chlorella pyrenoidosa*	10- to 14-day EC_{50} reduction in growth	8900 mg/litre	Stratton & Smith (1988)
Duckweed	*Lemna minor*	7-day IC_{25} growth inhibition	4900 mg/litre	Peterson et al. (1997)
Water flea	*Daphnia magna*	acute 48-h EC_{50} immobilization	14 500 mg/litre	Poirier et al. (1986)
Water flea	*Daphnia magna*	acute 48-h EC_{50} survival and mortality	15 700 mg/litre	Adams & Heidolph (1985)
Water flea	*Daphnia magna*	acute 48-h LC_{50} mortality	14 400 mg/litre	Ziegenfuss et al. (1986)
Water flea	*Daphnia magna*	acute 48-h LC_{50} mortality	14 530 mg/litre	Call et al. (1983)
Water flea	*Daphnia magna*	acute 48-h EC_{50} immobilization	13 100 mg/litre	Sebaugh et al. (1991)

Table 5 (contd).

Test species	Latin name	End-point	Range	References
Water flea	*Daphnia magna*	chronic 21-day EC_{50} survival and mortality	3721 mg/litre	Adams & Heidolph (1985)
Water flea	*Daphnia magna*	chronic 21-day NOEL/LOEC survival and mortality	1500–3000 mg/litre	Adams & Heidolph (1985)
Water flea	*Daphnia magna*	chronic 28-day NOEL survival and mortality	1140 mg/litre	Leblanc & Surprenant (1983)
Water flea	*Daphnia magna*	acute 48-h EC_{50} survival and mortality	12 400 mg/litre	Leblanc & Surprenant (1983)
Insect larvae	*Paratanytarsus parthenogeneticus*	48-h EC_{50}	36 200 mg/litre	Poirier et al. (1986)
Insect larvae	*Tanytarsus dissimilis*	48-h LC_{50}	36 000 mg/litre	Call et al. (1983)
Insect larvae	*Chironomus tentans*	acute 48-h LC_{50} mortality	33 500 mg/litre	Ziegenfuss et al. (1986)
Shrimp	*Crangon crangon*	LC_{50}	>100 mg/litre	Portmann & Wilson (1971)
Rainbow trout	*Oncorhynchus mykiss*	acute 96-h LC_{50} mortality	9800–12 000 mg/litre	Johnson & Finley (1980); Call et al. (1983); Poirier et al. (1986)
Winter flounder	*Pleuronectes americanus*	inhibition of the enzyme activity in intestinal mucosae	50 000 mg/litre	Janicki & Kinter (1971)
Zebrafish	*Brachydanio rerio*	acute 96-h LC_{50} mortality	8840 mg/litre	Groth et al. (1994)
Fathead minnow	*Pimephales promelas*	acute 96-h LC_{50} mortality	9080–11 400 mg/litre	Batchelder (1976); Call et al. (1983); Poirier et al. (1986)
Bluegill	*Lepomis macrochirus*	acute 96-h LC_{50} mortality	7100–7500 mg/litre	Call et al. (1983); Poirier et al. (1986)
Soil fungi	*Sclerotinia homeocarpa*	EC_{50} inhibition of fungal growth, compared with a control growth of 50–70 mm	4840 mg/litre	Stratton (1985)
Soil fungi	*Pythium ultimum*	EC_{50} inhibition of fungal growth, compared with a control growth of 50–70 mm	10 250 mg/litre	Stratton (1985)
Soil fungi	*Pestalotia* sp.	EC_{50} inhibition of fungal growth, compared with a control growth of 50–70 mm	5970 mg/litre	Stratton (1985)
Wheat and bean seeds		inhibition of germination	50 000 mg/litre	Szabo (1972)
Rat		2-year inhalational NOEL, 6 h/day, 5 days/week exposure changes in body weight and clinical chemistry parameters	75 mg/m^3	Malley et al. (1994)

[a] Personal communications from M. Harwood, Environment Canada, to A. Chevrier, Environment Canada, dated 2 and 5 December 1997.

Portmann & Wilson (1971) reported an LC_{50} of >100 mg/litre for *Crangon crangon*.

10.2 Terrestrial environment

There is little information available on the toxicity of DMF to terrestrial vascular plants. Szabo (1972) reported that DMF did not inhibit germination of wheat and bean seeds at 1% (approximately 10 000 mg/litre), but did at 5% (approximately 50 000 mg/litre); however, little methodological information is provided with which to assess the quality of the data. The IC_{25} of 4900 mg/litre for the duckweed (*Lemna minor*), an aquatic angiosperm, indicates that terrestrial angiosperms may not be sensitive to DMF (Peterson et al., 1997). The most sensitive organism in the terrestrial

compartment appears to be the soil fungus *Sclerotinia homeocarpa*, with an EC_{50} of 4840 mg/litre for growth inhibition (Stratton, 1985).

Although information on effects of DMF on wild-life has not been identified, a review of laboratory studies on experimental animals (WHO, 1991) concludes that acute toxicity of DMF in a variety of species is low. Only one chronic (2-year) inhalation assay was identified in recent literature (Malley et al., 1994). In that study, a LOEC of 25 ppm (75 mg/m^3) following inhalation of DMF was reported, based on changes in body weight and clinical chemistry.

11. EFFECTS EVALUATION

11.1 Evaluation of health effects

11.1.1 Hazard identification and dose–response assessment

11.1.1.1 Effects in humans

Consistent with the results of studies in experimental animals, available data from case reports and cross-sectional studies in occupationally exposed populations indicate that the liver is the target organ for the toxicity of DMF in humans. The profile of effects is consistent with that observed in experimental animals, with gastrointestinal disturbance, alcohol intolerance, increases in serum hepatic enzymes (AST, ALT, (-GT, and AP), and histopathological effects (hepatocellular necrosis, enlarged Kupffer cells, microvesicular steatosis, complex lysosomes, pleomorphic mitochondria, and fatty changes with occasional lipogranuloma) being observed. Effects observed at lowest concentrations in cross-sectional studies in occupationally exposed populations for which there is some information on dose–response are increases in serum hepatic enzymes.

Based on the limited data available, there is no convincing, consistent evidence of increased risk of cancer at any site associated with exposure to DMF in the occupational environment. Case reports of testicular cancers have not been confirmed in a cohort and case–control study. There have been no consistent increases in tumours at other sites associated with exposure to DMF.

There is also little consistent, convincing evidence of genotoxicity in populations occupationally exposed to DMF, with results of available studies of exposed workers (to DMF and other compounds) being mixed. The pattern of observations is not consistent with variations in exposure across studies. However, in view of the positive dose–response relationship observed in the one study in which it was investigated, this area may be worthy of additional work, although available data on genotoxicity in experimental systems are overwhelmingly negative.

11.1.1.2 Effects in experimental animals

DMF has low acute toxicity and is slightly to moderately irritating to the eyes and skin, based on limited data acquired in non-standard assays. Available data are inadequate as a basis for characterization of the potential of DMF to induce sensitization. In acute and repeated-dose toxicity studies, DMF has been consistently hepatotoxic, inducing effects on the liver at lowest concentrations or doses. The profile of effects includes alterations in hepatic enzymes, increases in liver weight, progressive degenerative histopathological changes and eventually cell death, and increases in serum hepatic enzymes. Species variation in sensitivity to these effects has been observed, with the order of sensitivity being mice > rats > monkeys.

Although the database for carcinogenicity is limited to two adequately conducted bioassays in rats and mice, there have been no increases in the incidence of tumours following chronic inhalation exposure to DMF. The weight of evidence for genotoxicity is overwhelmingly negative, based on extensive investigation in *in vitro* assays, particularly for gene mutation, and a more limited database *in vivo.*

DMF has induced adverse reproductive effects only at concentrations considerably greater than those associated with adverse effects on the liver. In adequately conducted and reported primarily recent developmental studies, fetotoxic and teratogenic effects have been consistently observed only at maternally toxic concentrations or doses.

Available data are inadequate as a basis for assessment of the neurological, immunological, or skin sensitizing effects of DMF.

The following guidance is provided as a possible basis for derivation of limits of exposure and judgement of the quality of environmental media by relevant authorities.

11.1.2 Criteria for setting tolerable concentrations or guidance values

In both humans and experimental animals exposed to DMF, the target organ has been the liver, consistent with local action of a reactive intermediate in the tissue where it is primarily metabolized. Available data indicate that there are considerable variations between experimental animals and humans in the proportion of

DMF metabolized by the putatively toxic pathway, with the resulting implication that humans may be more sensitive to the effects of DMF. Also, since there are data available to serve as the basis for at least crude characterization of exposure–response for parameters associated with hepatic toxicity in workers, the tolerable concentration (TC) is based on data on inhalation in humans, although it should be noted that these values do not account for likely additional exposure by dermal absorption. Analyses of dose–response for hepatic effects in the studies in experimental animals are presented for comparison. Since exposure in the general environment is likely to be primarily through air, emphasis in this section is on the generally more extensive database on toxicity by the inhalation route.

Effects on the liver observed at lowest concentration in cross-sectional studies in occupationally exposed populations for which there is some information on exposure–response are increases in serum hepatic enzymes. The results concerning exposure–response are consistent across studies, with increases in serum hepatic enzymes not being observed at concentrations in the range of 1–6 ppm (3–18 mg/m^3). At higher levels of exposure (>7 ppm [>21 mg/m^3]), increased serum levels of hepatic enzymes have been observed consistently. Cirla et al. (1984) reported significant increases in serum (-GT in 100 workers exposed to 7 ppm (21 mg/m^3). Similarly, Fiorito et al. (1997) reported significant increases in serum ALT, AST, (-GT, and AP in workers exposed to 7 ppm (21 mg/m^3).

Catenacci et al. (1984) did not observe differences between serum levels of SGOT, SGPT, and (-GT in workers employed for more than 5 years. In view of the small number of subjects exposed to the mean TWA of 6 ppm (18 mg/m^3) DMF (n = 28), negative results reported therein may be a function of lack of power of the study to detect a meaningful effect and are not, therefore, necessarily inconsistent with the results of Cirla et al. (1984) and Fiorito et al. (1997).

Based on the lowest-observed-adverse-effect level (LOAEL) of 7 ppm (21 mg/m^3), a TC[1] has been derived as follows:

$$TC = \frac{7\text{ppm}}{50} \times 8/24 \times 5/7$$

= 0.03 ppm (0.1 mg/m^3)

where:

- 7 ppm (21 mg/m^3) is the LOAEL for increases in serum hepatic enzymes in workers exposed primarily to DMF, reported by Cirla et al. (1984) and Fiorito et al. (1997); it should be noted that the observed small increases in a few serum hepatic enzymes are considered to be only minimally adverse, with associated hepatic damage likely being reversible upon cessation of exposure;
- 8/24 and 5/7 are the factors to convert exposure during 8 h/day and 5 days/week, respectively, to continuous exposure;
- 50 is the uncertainty factor (×10 for intraspecies [interindividual][2] variation, including sensitive subgroups; ×5 to account primarily for less than lifetime exposure; although the TC is based on a LOAEL, observed effects are considered to be only minimally adverse).

Although not the basis of the TC developed here, there are several important observations from dose–response analyses of the results of the studies in animals (see Appendix 4). The lowest reported benchmarks for a range of hepatic effects in rats and mice following inhalation are those for histopathological lesions in the liver of both rats and mice, which are higher but in the same range as those reported to induce effects on hepatic function in the studies in workers. It should be noted, though, that, due to the nature of the effects on which they were based (increases in serum hepatic enzymes versus histological effects), the benchmarks in humans are not strictly comparable.

It is also evident that there is progression of effects from the medium-term to long-term studies, with effects being more severe following long-term exposure (although quantitative values for the lowest benchmarks for different types of lesions in the medium-term and long-term studies are similar).

11.1.3 Sample risk characterization

Due to the nature of use, patterns of release, and environmental fate of DMF, the focus of the human health risk characterization for indirect exposure is populations exposed through air in the vicinity of industrial point sources.

With a reported annual loading of less than 20 tonnes and generally less than 1 tonne at any location in the sample country (i.e., Canada), continuous releases of consistent magnitude likely result in long-term expo-

[1] The term “tolerable concentration” is used here in the same sense as the term “tolerable intake” as defined by IPCS (1994), i.e., “an estimate of the intake of a substance over a lifetime that is considered to be without appreciable health risk.”

[2] Available quantitative data are insufficient to replace default values for the component of this uncertainty factor with data-derived values (IPCS, 1994).

sure to small concentrations (worst-case estimate in Canada, 0.11 mg/m^3) of DMF near point sources. Because of the absence of empirical data on concentrations of DMF in air in Canada, an estimated exposure value (EEV) was calculated based on release data for the largest Canadian emitter, making several conservative assumptions.

The largest annual release reported at one location can be expressed on a daily basis (12.7 tonnes/year = 0.0348 tonnes/day or 3.48×10^7 mg/day). As a conservative estimate, it will be assumed that daily releases of DMF are contained within a cylinder having a radius of 1 km centred on the point source. Dispersion within 1 km is likely a conservative assumption for a number of reasons. First, the greatest reported emissions are occurring in a mixed industrial and agricultural area (Environment Canada, 1999b). The site is paved with asphalt; as such, wild plants and mammals will not likely be found in the immediate vicinity of the source. Finally, although the specific dispersal behaviour of DMF has not been documented near the source, results of dispersion modelling indicate that concentrations of other contaminants released to air elsewhere tend to decrease rapidly within a few kilometres of industrial point sources (e.g., Davis, 1997; Thé, 1998).

Upward movement of organic compounds generally does not exceed 100 m at night and may exceed 1000 m during the day.[1] The more conservative value of 100 m will be used as a ceiling for estimating the exposure concentrations throughout the day.

This provides a dispersal volume of 3.14×10^8 m^3 in the form of a cylinder 100 m in height and 1 km in radius. With a daily release of 3.48×10^7 mg/day, the daily increase in the concentration of DMF in air is estimated at 0.11 mg/m^3. Since ambient levels in the cylinder are likely to be lower than this daily increase of 0.11 mg/m^3, it will be used as a conservative EEV. Reaction with hydroxyl radicals will tend to reduce the concentrations of DMF in the daytime. Since the degradation half-life of DMF could be a week or more, continuous daily inputs would lead to buildup of DMF within the cylinder in the absence of any other loss process. However, fugacity-based modelling suggests that advection processes, i.e., rain and wind, are the major factors in determining concentrations in the atmosphere. Even under essentially stagnant conditions, with a wind speed of 1 km/h, the rate of advection of DMF out of the cylinder is so fast that the steady-state concentration would be 0.01 mg/m^3 or less. At a typical average wind speed of 10 km/h, the concentration of DMF in the cylinder would be reduced by a factor of approximately 100. The EEV of 0.11 mg/m^3 is generally higher than or comparable to measurements made in other countries.

Worst-case estimates of airborne levels in the immediate vicinity of the largest emitter in the sample country (0.11 mg/m^3), which are likely 10- to 100-fold greater than those anticipated under most conditions, do not appreciably exceed the TC (0.1 mg/m^3) derived on the basis of increases in serum hepatic enzymes in exposed workers.

11.1.4 Uncertainties and degree of confidence in human health risk characterization

Quantitative estimates of ambient levels of DMF in the vicinity of point sources in the sample country on which the human health risk characterization is based are highly uncertain (see discussion of uncertainty in section 11.2.3) and likely conservative, although consistent with highest consentrations measured in other countries. The proximity of these predicted concentrations in the vicinity of point sources to residential areas is also unknown. Available monitoring data are inadequate as a basis for characterization of the exposure of the general population to DMF.

There is a high degree of confidence based on studies in both humans and experimental animals that the liver is the target organ for the toxicity of DMF. Cross-sectional studies on hepatic effects in workers, limited principally to males, were complicated by co-exposures to other substances and limitations of available data on exposure, including, in some cases, lack of monitoring data for individuals. However, the levels that induced minimally adverse effects were remarkably consistent across a large number of studies. The TC developed on the basis of increases in serum hepatic enzymes in occupationally exposed populations is likely conservative, since it does not take into account additional exposure by the dermal route.

Although cases of testicular cancer among people exposed to DMF have been reported, these findings have not been corroborated in (limited) epidemiological studies, and it is thus unlikely that DMF is carcinogenic to humans.

11.2 Evaluation of environmental effects

11.2.1 Terrestrial assessment end-points

Since most DMF appears to be released to air in the sample country, and based on the fate of DMF in the ambient environment, biota are expected to be exposed to DMF primarily in air; little exposure to DMF from surface water, soil, or benthic organisms is expected. Based on this, and because of the low toxicity of DMF to

[1] Notes from N.J. Bunce, University of Guelph, Guelph, Ontario, to A. Chevrier, Environment Canada, dated 1 June 1998.

a wide range of aquatic and soil organisms, it is unlikely that organisms will be exposed to harmful levels of DMF in Canadian surface waters, soils, or groundwater. Therefore, the focus of the environmental risk characterization will be on terrestrial organisms exposed directly to DMF in ambient air.

Terrestrial plants can be exposed to DMF by direct contact with the atmosphere, but also conceivably by diffusion from raindrops deposited on leaves. No data are available on the toxicity of DMF to terrestrial vascular plants. Seeds, soil fungi, and aquatic angiosperm macrophytes can be used as indicators of the potential sensitivities of trees, shrubs, and other plants. The most sensitive of these organisms appears to be the soil fungus *Sclerotinia homeocarpa*, with an EC_{50} of 4840 mg/litre for growth inhibition (Stratton, 1985). In view of the generally high effect concentrations, it is unlikely that terrestrial plants are particularly sensitive to DMF.

As most DMF is released to air and bioaccumulation is not expected, effects on wildlife will occur mainly through direct exposure by inhalation in the vicinity of the point source. Based on the available information, the home range of common small to medium-sized eastern Canadian mammals is generally much less than 1 km^2 (Banfield, 1974; Burt & Grossenheider, 1976; Forsyth, 1985; US EPA, 1999). By contrast, the home range of the raccoon, a common suburban visitor, is quite variable in size, reportedly ranging from a few square kilometres to thousands of square kilometres (Burt & Grossenheider, 1976; US EPA, 1999). Therefore, small mammals are likely exposed over long periods to highest concentrations of DMF within a few kilometres of the site, while the more mobile medium-sized mammals are probably exposed over time to lower average levels of DMF.

No information has been found on effects of DMF on wildlife. Experimental animals used in laboratory studies will be used as surrogates for small and medium-sized mammals exposed to DMF through inhalation.

11.2.2 Sample environmental risk characterization

The calculation of the EEV is presented in section 11.1.3.

Analysis of exposure pathways and subsequent identification of sensitive receptors are the basis for selection of environmental assessment end-points (e.g., adverse reproductive effects on sensitive fish species in a community). For each end-point, a conservative EEV is selected and an estimated no-effects value (ENEV) is determined by dividing a critical toxicity value (CTV) by an application factor. A hyperconservative or conservative quotient (EEV/ENEV) is calculated for each of the assessment end-points in order to determine whether there is potential ecological risk.

The long-term (18-month) inhalation LOAEC of 75 mg/m^3 measured for mice is used as a CTV for exposure of small mammals. This value was selected from a large data set composed of acute and long-term studies conducted on a number of laboratory species. Although no direct effects related to survival were observed at the exposure concentrations (up to 1200 mg/m^3), nor were any haematological changes or effects on the estrous cycle observed, the incidence of hepatocellular hypertrophy, hepatic single-cell necrosis, and hepatic Kupffer cell hyperplasia/pigment accumulation was increased at 75 mg/m^3 (Malley et al., 1994). Such effects may not directly manifest themselves as population-level effects in wildlife species; therefore, the ENEV is derived by dividing the CTV by a reduced application factor of 5. This factor also accounts for the extrapolation from a low-effect level to a no-effect level, as well as the uncertainty surrounding the extrapolation from laboratory to field conditions and interspecies and intraspecies variations in sensitivity. As a result, the ENEV is 15 mg/m^3. Therefore, using the EEV of 0.11 mg/m^3, the quotient EEV/ENEV= 0.007. Since this conservative quotient is less than 1, it is unlikely that DMF causes adverse effects on terrestrial organisms in the sample country.

11.2.3 Discussion of uncertainty

There are a number of potential sources of uncertainty in this environmental risk assessment.

The calculated Henry's law constant is uncertain, as solubility cannot be measured. Based on sensitivity analysis, the fugacity-based partitioning estimates can be sensitive to the value used as the Henry's law constant.[1]

Ambient levels near Canadian sources are not available. The EEV was therefore estimated based on available information on releases. This calculated EEV is, however, generally consistent with the highest concentrations measured in other countries. It is unlikely that there are concentrations of DMF in the sample country that are higher than those calculated and used in this assessment. For air, reported releases at the selected location by far exceed reported releases to air at any other location and as such likely constitute a worst-case scenario. For water, concentrations are expected to

[1] Collection of notes and modelling results submitted by A. Bobra, AMBEC Environmental Consultant, to Chemicals Evaluation Division, Commercial Chemicals Evaluation Branch, Environment Canada, 1999.

be low because of the limited releases identified to this medium and the limited partitioning of DMF from air into water. Small spills and leakage could increase levels of DMF in soil and groundwater; however, the available information suggests that such releases would be small and infrequent.

Regarding effects of DMF on terrestrial organisms, although no toxicity data were identified for vascular plants, data for effects on seeds and aquatic macrophytes suggest that terrestrial vegetation is not particularly sensitive to DMF. Additional evidence of effects on terrestrial plants would strengthen the conclusion that DMF is not expected to damage gymnosperms, angiosperms, and other vascular plants.

There is uncertainty concerning the extrapolation from available toxicity data for laboratory mammals to potential effects on wildlife populations. To account for these uncertainties, an application factor was used in the environmental risk analysis to derive ENEVs.

12. PREVIOUS EVALUATIONS BY INTERNATIONAL BODIES

IARC (1999) has classified DMF in Group 3 (not classifiable as to its carcinogenicity to humans). There was inadequate evidence for carcinogenicity of DMF in humans. There was evidence suggesting lack of carcinogenicity of DMF in experimental animals.

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APPENDIX 1 — SOURCE DOCUMENT

Government of Canada (in press)

Copies of the *Canadian Environmental Protection Act Priority Substances List Assessment Report* (Government of Canada, in press) and unpublished supporting documentation for *N,N*-dimethylformamide may be obtained from:

Commercial Chemicals Evaluation Branch
Environment Canada
14th floor, Place Vincent Massey
351 St. Joseph Blvd.
Hull, Quebec
Canada K1A 0H3

or

Environmental Health Centre
Health Canada
Address Locator: 0801A
Tunney's Pasture
Ottawa, Ontario
Canada K1A 0L2

Initial drafts of the supporting documentation and Assessment Report for DMF were prepared by staff of Health Canada and Environment Canada.

The environmental sections were reviewed externally by:

D. Andrews, Golder Associates Ltd.
K. Bolton, University of Toronto
N. Bunce, University of Guelph
R. Gensemer, Boston University
D. Hastie, York University
S. Mabury, University of Toronto
M. Mumtaz, Chinook Group Ltd.
C. Nalewajko, University of Toronto
M. Sheppard, EcoMatters Inc.

Sections of the supporting documentation pertaining to human health were reviewed externally by G. Kennedy, DuPont Haskell Laboratory for Toxicology and Industrial Medicine, to address adequacy of coverage.

Accuracy of reporting, adequacy of coverage, and defensibility of conclusions with respect to hazard identification and dose–response analyses were considered at a panel of the following members, convened by Toxicology Excellence in Risk Assessment on 14 February 2000 in Ottawa, Canada:

M.S. Abdel-Rahman, University of Medicine & Dentistry of New Jersey
C. Abernathy, US Environmental Protection Agency
J.P. Christopher, California Environmental Protection Agency
J.C. Collins, Solutia, Inc.
J.T. Colman, Syracuse Research Corporation
M. Mumtaz, Agency for Toxic Substances and Disease Registry
K.A. Poirier, Toxicology Excellence in Risk Assessment
J.E. Whalen, US Environmental Protection Agency

APPENDIX 2 — CICAD PEER REVIEW

The draft CICAD on *N,N*-dimethylformamide was sent for review to institutions and organizations identified by IPCS after contact with IPCS National Contact Points and Participating Institutions, as well as to identified experts. Comments were received from:

A. Aitio, International Programme on Chemical Safety, World Health Organization, Switzerland

M. Baril, Institut de Recherche en Santé et en Sécurité du Travail du Québec (IRSST), Canada

R. Benson, Drinking Water Program, US Environmental Protection Agency, USA

R.S. Chhabra, National Institute for Environmental and Health Sciences/National Institutes of Health (NIEHS/NIH), USA

R. Hertel, Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Germany

C. Hiremath, US Environmental Protection Agency, USA

H. Kafferlein, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander University Erlangen-Nuremberg, Germany

F. Larese, Institute of Occupational Medicine, University of Trieste, Italy

H. Lendle, Product Safety, BASF AG, Germany

I. Mangelsdorf, Fraunhofer Institute for Toxicolgy and Aerosol Research, Germany

J. Mraz, Centre of Industrial Hygiene and Occupational Diseases, National Institute of Public Health, Czech Republic

P. Ridgeway, Health and Safety Executive, United Kingdom

P. Schulte, National Institute for Occupational Safety and Health, USA

E. Soderlund, Department of Environmental Medicine, National Institute of Public Health, Norway

D. Willcocks, National Industrial Chemicals Notification and Assessment Scheme (NICNAS), Australia

P. Yao, Chinese Academy of Preventive Medicine, People's Republic of China

K. Ziegler-Skylakakis, Beratergremium für Umweltrelevante Altstoffe (BUA), Germany

APPENDIX 3 — CICAD FINAL REVIEW BOARD

Helsinki, Finland, 26–29 June 2000

Members

Mr H. Ahlers, Education and Information Division, National Institute for Occupational Safety and Health, Cincinnati, OH, USA

Dr T. Berzins, National Chemicals Inspectorate (KEMI), Solna, Sweden

Dr R.M. Bruce, Office of Research and Development, National Center for Environmental Assessment, US Environmental Protection Agency, Cincinnati, OH, USA

Mr R. Cary, Health and Safety Executive, Liverpool, United Kingdom *(Rapporteur)*

Dr R.S. Chhabra, General Toxicology Group, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Dr H. Choudhury, National Center for Environmental Assessment, US Environmental Protection Agency, Cincinnati, OH, USA

Dr S. Dobson, Centre for Ecology and Hydrology, Monks Wood, Abbots Ripton, United Kingdom *(Chairman)*

Dr H. Gibb, National Center for Environmental Assessment, US Environmental Protection Agency, Washington, DC, USA

Dr R.F. Hertel, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin, Germany

Ms K. Hughes, Priority Substances Section, Environmental Health Directorate, Health Canada, Ottawa, Ontario, Canada

Dr G. Koennecker, Chemical Risk Assessment, Fraunhofer Institute for Toxicology and Aerosol Research, Hanover, Germany

Ms M. Meek, Existing Substances Division, Environmental Health Directorate, Health Canada, Ottawa, Ontario, Canada

Dr A. Nishikawa, Division of Pathology, Biological Safety Research Centre, National Institute of Health Sciences, Tokyo, Japan

Dr V. Riihimäki, Finnish Institute of Occupational Health, Helsinki, Finland

Dr J. Risher, Agency for Toxic Substances and Disease Registry, Division of Toxicology, US Department of Health and Human Services, Atlanta, GA, USA

Professor K. Savolainen, Finnish Institute of Occupational Health, Helsinki, Finland *(Vice-Chairman)*

Dr J. Sekizawa, Division of Chem-Bio Informatics, National Institute of Health Sciences, Tokyo, Japan

Dr S. Soliman, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

Ms D. Willcocks, National Industrial Chemicals Notification and Assessment Scheme, Sydney, NSW, Australia

Observer

Dr R.J. Lewis (representative of European Centre for Ecotoxicology and Toxicology of Chemicals), Epidemiology and Health Surveillance, ExxonMobil Biomedical Sciences, Inc., Annandale, NJ, USA

Secretariat

Dr A. Aitio, International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland *(Secretary)*

Dr P.G. Jenkins, International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland

Dr M. Younes, International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland

APPENDIX 4 — BENCHMARK DOSE CALCULATIONS

In subchronic inhalation assays in F344 rats, there was an increase in relative liver weight in females and increased cholesterol in both sexes at 50 ppm (150 mg/m³), with no clear dose–response (LOEC) (NTP, 1992a), progressive histopathological hepatic changes in both sexes at 400 and 800 ppm (1200 and 2400 mg/m³) (Craig et al., 1984), and hepatocellular necrosis in both sexes at 400 ppm (1200 mg/m³) (NTP, 1992a). B6C3F1 mice had hepatocellular hypertrophy at 50 ppm (150 mg/m³) (LOEC), in addition to significantly increased relative liver weight in both sexes without clear dose–response (NTP, 1992a) and hepatic cytomegaly at 150 ppm (450 mg/m³) and higher (Craig et al., 1984). No signs of toxicity were observed in monkeys exposed to up to 500 ppm (1500 mg/m³) (Hurtt et al., 1992).

In a chronic inhalation bioassay in Crl:CD BR rats, at 100 ppm (300 mg/m³), there were significant increases in centrilobular hepatocellular hypertrophy (both sexes), hepatic accumulation of lipofuscin/haemosiderin (both sexes), and hepatic single-cell necrosis (females only). In mice [Crl:CD 1 (ICR)BR], at 25 ppm (75 mg/m³), there was centrilobular hepatocellular hypertrophy (males), hepatic single-cell necrosis (males and females), and hepatic Kupffer cell hyperplasia/pigment accumulation (males) (Malley et al., 1994).

Data on dose–response following ingestion are limited to medium-term exposure studies. At 250 mg/kg body weight per day, liver cell enlargement was reported in Crl:CD rats; at 50 mg/kg body weight per day, relative liver weight was significantly increased in males (Kennedy & Sherman, 1986). In Wistar rats, relative liver weight was significantly increased at 69 mg/kg body weight per day, but no histopathological lesions were observed at doses up to 235 mg/kg body weight per day (Becci et al., 1983). In CD-1 mice, only mild histopathological changes were observed in the liver at 246 mg/kg body weight per day; at 96 mg/kg body weight per day, relative liver weight was significantly increased in females. No adverse effects were observed in beagle dogs administered up to 34.8 mg/kg body weight day in the diet for 13 weeks.

It should be noted that the lowest concentration (50 ppm [150 mg/m³]) at which effects were observed in the liver of rats (NTP, 1992a) in an inhalation assay is equivalent to an intake of 46.5 mg/kg body weight per day in rats,[1] which is consistent with the effects levels in Crl:CD rats (Kennedy & Sherman, 1986) and Wistar rats (Becci et al., 1983) following dietary exposure. The lowest concentration (50 ppm [150 mg/m³]) to which mice were exposed in the NTP (1992a) is equivalent to an intake of 200 mg/kg body weight per day,[2] which is consistent with the effect levels in the dietary assay in mice reported by Becci et al. (1983).

Reported incidence, benchmark concentrations (BMCs) at the 5% level, and associated *P*-values and goodness of fit statistics for effects on the liver for relevant end-points in the most robust medium- and long-term exposure studies for ingestion and inhalation, respectively, are presented in Tables 2 and 3.

For the discrete end-points, the BMC_{05} is defined as the concentration of chemical that causes a 5% increase in incidence over the background response rate. It is calculated by first fitting the following model to the dose–response data (Howe, 1995):

$$P(d) = q_0 + (1 - q_0)[1 - e^{-q_1 d - \ldots - q_k d^k}]$$

where d is dose, k is the number of dose groups in the study, $P(d)$ is the probability of the animal developing the effect at dose d, and $q_i > 0$, $i = 1,\ldots, k$ is a parameter to be estimated.

The models were fit to the incidence data using THRESH (Howe, 1995), and the BMC_{05}s were calculated as the concentration C that satisfies

$$\frac{P(C) - P(0)}{1 - P(0)} = 0.05$$

A chi-square lack of fit test was performed for each of the model fits. The degrees of freedom for this test are equal to k minus the number of q_i's whose estimates are non-zero. A *P*-value less than 0.05 indicates a significant lack of fit.

For the continuous end-points, the BMC_{05} is defined as the dose that causes a 5% increase in the absolute risk of seeing an "adverse" response. This method utilizes the "hybrid" method of Crump (1995), in which the adverse response level in the control group is specified as 5%. That is, 5% of the animals in the control group would, by natural variation, have a response that would be considered adverse. Then, the probability of being adverse, as opposed to the response itself, is modelled.

The Weibull model was fit to each of the end-points using BENCH_C (Crump & Van Landingham, 1996):

$$P(d) = p_0 + (1 - p_0)[1 - e^{(-\beta d)^k}]$$

where d is dose, $P(d)$ is the probability of an adverse response at dose d, and k, β, and p_0 are parameters to be estimated. The BMC_{05} was then calculated as the concentration C such that

$$P(C) - P(0) = 0.05$$

An F-test was used to assess lack of fit of the model. A *P*-value less than 0.05 indicates lack of fit.

[1] 1 mg/m³ = 0.31 mg/kg body weight per day in rats (Health Canada, 1994).

[2] 1 mg/m³ = 1.33 mg/kg body weight per day in mice (Health Canada, 1994).

N,N-DIMETHYLFORMAMIDE

0457
October 2000

CAS No: 68-12-2
RTECS No: LQ2100000
UN No: 2265
EC No: 616-001-00-X

Dimethylformamide
DMF
DMFA
N-formyldimethylamine
C_3H_7NO / $HCON(CH_3)_2$
Molecular mass: 73.09

TYPES OF HAZARD/ EXPOSURE	ACUTE HAZARDS/SYMPTOMS	PREVENTION	FIRST AID/FIRE FIGHTING
FIRE	Flammable. Gives off irritating or toxic fumes (or gases) in a fire.	NO open flames, NO sparks, and NO smoking. NO contact with oxidizing agents.	Powder, alcohol-resistant foam, water spray, carbon dioxide.
EXPLOSION	Above 58°C explosive vapour/air mixtures may be formed.	Above 58°C use a closed system, ventilation.	In case of fire: keep drums, etc., cool by spraying with water.
EXPOSURE		**PREVENT GENERATION OF MISTS! AVOID EXPOSURE OF (PREGNANT) WOMEN!**	
Inhalation	Abdominal pain. Diarrhoea. Nausea. Vomiting. Facial flushing.	Ventilation, local exhaust, or breathing protection.	Fresh air, rest. Refer for medical attention.
Skin	MAY BE ABSORBED!	Protective gloves. Protective clothing.	Remove contaminated clothes. Rinse and then wash skin with water and soap. Refer for medical attention.
Eyes	Redness. Pain.	Safety goggles, or eye protection in combination with breathing protection.	First rinse with plenty of water for several minutes (remove contact lenses if easily possible), then take to a doctor.
Ingestion		Do not eat, drink, or smoke during work.	Rinse mouth.

SPILLAGE DISPOSAL	PACKAGING & LABELLING
Ventilation. Remove all ignition sources. Collect leaking and spilled liquid in sealable containers as far as possible. Absorb remaining liquid in sand or inert absorbent and remove to safe place. (Extra personal protection: complete protective clothing including self-contained breathing apparatus).	T Symbol R: 61-20/21-36 S: 53-45 Note: E UN Hazard Class: 3 UN Pack Group: III

EMERGENCY RESPONSE	STORAGE
Transport Emergency Card: TEC (R)-30G35 NFPA Code: H1; F2; R0	Separated from strong oxidants, halogens.

IPCS
International Programme on Chemical Safety

Prepared in the context of cooperation between the International Programme on Chemical Safety and the European Commission

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0457 **N,N-DIMETHYLFORMAMIDE**

IMPORTANT DATA

Physical State; Appearance
COLOURLESS TO YELLOW LIQUID, WITH CHARACTERISTIC ODOUR.

Chemical dangers
The substance decomposes on heating or on burning producing toxic fumes including nitrogen oxides.
Reacts violently with oxidants, nitrates and halogenated hydrocarbons. Attacks some plastic and rubber.

Occupational exposure limits
TLV: 10 ppm; (skin) (ACGIH 2000).
MAK: 10 ppm; 30 mg/m^3; skin, Re2 (1999)

Routes of exposure
The substance can be absorbed into the body by inhalation and through the skin.

Inhalation risk
A harmful contamination of the air will be reached rather slowly on evaporation of this substance at 20°C.

Effects of short-term exposure
The substance is irritating to the eyes.
The substance may cause effects on the liver, resulting in jaundice. See Notes.

Effects of long-term or repeated exposure
The substance may have effects on the liver, resulting in impaired functions.
Animal tests show that this substance possibly causes toxic effects upon human reproduction.

PHYSICAL PROPERTIES

Boiling point: 153°C
Melting point: -61°C
Relative density (water = 1): 0.95
Solubility in water: miscible
Vapour pressure, Pa at 25°C: about 492
Relative vapour density (air = 1): 2.5
Relative density of the vapour/air-mixture at 20°C (air = 1): 1.00
Flash point: 58°C c.c.
Auto-ignition temperature: 445°C
Explosive limits, vol% in air: 2.2-15.2 at 100°C
Octanol/water partition coefficient as log Pow: -0.87

ENVIRONMENTAL DATA

NOTES

Use of alcoholic beverages enhances the harmful effect.
Resulting symptoms could be delayed from several hours up to several days.
Environmental effects from the substance have been investigated, but none has been found.

ADDITIONAL INFORMATION

RÉSUMÉ D'ORIENTATION

Ce CICAD sur le *N*,*N*-diméthylformamide (DMF) a été préparé conjointement par la Direction de l'hygiène du milieu de Santé Canada et la Direction de l'évaluation des produits chimiques commerciaux d'Environnement Canada, sur la base d'une documentation préparée simultanément dans le cadre du Programme d'évaluation des substances prioritaires, en application de la Loi canadienne sur la protection de l'environnement (LCPE). Les évaluations sanitaires des substances prioritaires effectuées en application de cette loi portent sur les effets que pourraient avoir ces produits sur la santé humaine en cas d'exposition indirecte dans l'environnement. L'exposition professionnelle n'est pas abordée dans le document de base. La présente mise au point prend en compte les données sur les effets environnementaux jusqu'à septembre 1999 et les données sur les effets sanitaires jusqu'à février 2000. L'appendice 1 donne des informations sur la nature de l'examen par des pairs et sur les sources documentaires. D'autres études ont également été utilisées, à savoir celle l'IARC/CIRC (1999) et celle du BUA (1994). Des renseignements sur l'examen par des pairs du présent CICAD sont donnés à l'appendice 2. Ce CICAD a été adopté en tant qu'évaluation internationale lors de la réunion du Comité d'évaluation finale qui s'est tenue à Helsinki du 26 au 29 juin 2000. La liste des participants à cette réunion figure à l'appendice 3. La fiche internationale sur la sécurité chimique (ICSC 0457) du *N*,*N*-diméthylformamide, établie par le Programme international sur la sécurité chimique (IPCS, 1999), est également reproduite dans le présent document.

Le *N*,*N*-diméthylformamide (No CAS 68-12-2) est un solvant organique produit en grande quantité dans l'ensemble du monde. On utilise dans l'industrie chimique comme solvant, comme intermédiaire ou comme additif. Il se présente sous la forme d'un liquide incolore dégageant une faible odeur qui rappelle celle des amines. Il est miscible en toutes proportions à l'eau et à la plupart des solvants organiques. Sa tension de vapeur est relativement faible.

Une fois libéré dans l'air, le DMF y demeure en majeure partie jusqu'à décomposition par réaction avec des radicaux hydroxyles. La libération indirecte de DMF dans l'air, notamment à partir d'autres milieux, ne contribue guère au maintien de la concentration de ce composé dans le compartiment atmosphérique. On estime que le DMF présent dans l'air est photo-oxydé en l'espace de quelques jours. Une partie du DMF atmosphérique peut cependant atteindre le milieu aquatique ou terrestre, vraisemblablement à la faveur des précipitations. Le DMF qui passe dans l'eau subit une décomposition *in situ* sans transfert vers d'autres compartiments. Libéré dans le sol, il y demeure en majeure partie - probablement dans l'eau des pores - jusqu'à dégradation par voie chimique ou biologique. En cas de décharge dans les eaux ou au sol, on peut s'attendre à une biodégradation relativement rapide (demi-vie de 18 à 36 h). Si le composé parvient jusqu'aux nappes souterraines, sa décomposition anaérobie sera lente. Compte tenu du mode d'utilisation du DMF, l'exposition de la population générale à ce composé est vraisemblablement très faible.

Etant donné que dans le pays témoin, la majeure partie du DMF est effectivement libérée dans l'air et compte tenu du devenir de ce composé dans l'environnement, l'exposition des organismes vivants est essentiellement atmophérique et les organismes benthiques, comme ceux qui peuplent les eaux de surface ou le sol, sont sans doute peu exposés. Compte tenu de cela et étant donné la faible toxicité du DMF pour nombre d'organismes aquatiques ou terricoles, la caractérisation du risque vise essentiellement les organismes terrestres directement exposés au DMF présent dans l'air ambiant.

Le DMF est rapidement absorbé en cas d'exposition par voie orale, percutanée ou respiratoire. Une fois absorbé, le composé se répartit de façon uniforme dans l'organisme et après avoir été métabolisé principalement au niveau du foie, il est assez rapidement excrété par la voie urinaire sous la forme de métabolites. La principale voie métabolique consiste en une hydroxylation du groupement méthyle conduisant au *N*-(hydroxyméthyl)-*N*-méthylformamide (HMMF), qui est le principal métabolite urinaire chez l'Homme et l'animal. Le HMMF peut à son tour subir une décomposition en *N*-méthylformamide (NMF), dont l'hydroxylation enzymatique au niveau du groupement *N*-méthyle va entraîner la formation de *N*-(hydroxyméthyl)formamide (HMF), qui se décompose ensuite en formamide. Il existe également une possibilité de bifurcation métabolique à partir du NMF qui consiste en une oxydation du groupement formyle conduisant à la *N*-acétyl-*S*-(*N*-méthylcarbamoyl)cystéine (AMCC), métabolique dont on a décelé la présence dans l'urine humaine et l'urine de rongeurs. Au cours de ce processus, il se forme également un intermédiaire réactif dont la structure n'est pas encore élucidée (peut-être de l'isocyanate de méthyle). Bien qu'on ne dispose pas de preuve expérimentale directe, il se pourrait que ce composé soit le métabolite présumé toxique. A la lumière des données existantes, il semblerait que chez l'Homme, la proportion de DMF métabolisée par la voie présumée toxique soit plus importante que chez l'animal de laboratoire. Il existe une interaction métabolique entre le DMF et l'alcool, qui, bien qu'encore mal élucidée, pourrait être due à l'action inhibitrice de ce composé sur l'alcool-déshydrogénase.

Les données tirées d'analyses de cas individuels ou d'études transversales sur des populations professionnellement exposées, montrent, en accord avec

les résultats de l'expérimentation animale, que chez l'Homme, c'est le foie qui est l'organe-cible du DMF. L'ensemble des effets correspond à ce qui s'observe chez l'animal de laboratoire, c'est-à-dire des troubles digestifs, une intolérance à l'alcool, l'augmentation du taux sérique des enzymes hépatiques (aspartate-amino-transférase, alanine-aminotransférase, γ-glutamyl-transpeptidase et phosphatase alcaline) accompagnés d'anomalies histopathologiques et de modifications ultrastructurales (nécrose hépatocellulaire, hypertrophie des cellules de Kupffer, stéatose microvésiculaire, lysosomes complexes, mitochondries pléomorphes et dégénérescence graisseuse avec présence occasionnelle de lipogranulomes).

A la lumière des données disponibles, il n'existe pas de faits probants ni cohérents qui témoignent d'une augmentation des tumeurs de toutes localisations imputable à l'exposition au DMF sur le lieu de travail. Les cas de cancer du testicule qui avaient été rapportés n'ont pas été confirmés par une étude de cohorte cas-témoins. Pour ce qui est d'autres localisations, aucune augmentation systématique de la fréquence tumorale n'a pu être associée à une exposition au DMF.

En ce qui concerne la génotoxicité du composé pour des populations professionnellement exposées, les données ne sont pas non plus très probantes ni cohérentes, les résultats des études effectuées sur des travailleurs exposés (au DMF et à d'autres composés) étant mitigés. L'ensemble des observations ne cadre pas avec les variations de l'exposition d'une étude à l'autre. Cependant, en raison de la relation dose-réponse positive observée lors de l'étude où cette possibilité avait été explorée, il s'agit là d'un domaine qui mériterait des études supplémentaires, même si les résultats obtenus dans des systèmes d'épreuve expérimentaux sont très largement négatifs en ce qui concerne la génotoxicité du DMF.

La toxicité aiguë du DMF est faible et il n'est que légèrement à modérément irritant pour les yeux et la peau. On n'a pas trouvé de données sur son pouvoir sensibilisateur. Les études de toxicité aiguë ou chronique par administration de doses répétées mettent invariablement en évidence l'hépatotoxicité du DMF, même aux concentrations ou aux doses les plus faibles. Au nombre des effets constatés figurent des modifications touchant les enzymes hépatiques qui sont caractéristiques d'une action toxique, l'augmentation du poids du foie, une dégénérescence histopathologique progressive pouvant conduire à la mort cellulaire et l'accroissement du taux sérique des enzymes hépatiques. Après avoir exposé des rats et des souris par la voie respiratoire et la voie orale, on a constaté l'existence d'une relation dose-réponse pour l'ensemble de ces effets. Par ailleurs, l'ordre de sensibilité des diverses espèces relativement à ces effets est le suivant : souris > rat > singe.

La base de données relative à la cancérogénicité du DMF ne comporte en tout et pour tout que deux épreuves biologiques sur le rat et la souris, mais il en ressort néanmoins que l'inhalation prolongée de ce composé n'entraîne pas d'augmentation de l'incidence tumorale. Comme on l'a vu, les résultats des tests de génotoxicité sont très largement négatifs; ils proviennent d'études approfondies *in vitro*, consistant notamment à rechercher la présence de gènes mutés, ainsi que d'une base de données plus limitée constituée à partir d'épreuves *in vivo.*

L'expérimentation animale montre que le DMF n'a d'effets nocifs sur la reproduction qu'à des concentrations plus fortes que celles qui sont hépatotoxiques, après exposition tant par la voie respiratoire que par la voie orale. De même, lors d'études sur le développement bien conduites et publiées tout récemment, on n'a observé d'effets foetotoxiques et tératogènes systématiques qu'aux doses ou aux concentrations toxiques pour la mère.

Les données existantes sont insuffisantes pour permettre une évaluation des effets neurologiques et immunologiques du DMF.

Le présent CICAD et la caractérisation du risque que constitue le DMF ont essentiellement pour objet les effets de ce composé lors d'une exposition indirecte dans l'environnement.

C'est l'air au voisinage de sources ponctuelles de DMF qui fait courir à la population générale le risque d'exposition le plus important. D'après les études épidémiologiques effectuées sur des travailleurs exposés et les informations tirées de la base de données relativement fournie qui a été constituée à partir des résultats de l'expérimentation animale, c'est le foie qui constitue l'organe cible de l'action toxique du DMF. En se basant sur l'augmentation du taux sérique des enzymes hépatiques, on a fixé à 0,03 ppm (0,1 mg/m^3) la concentration tolérable.

On n'a pas trouvé de données sur la toxicité du DMF pour les plantes vasculaires terrestres. Pour les indicateurs de sensibilité potentielle des arbres et des arbustes, les concentrations agissantes sont élevées, aussi est-il peu probable que les végétaux terrestres soient particulièrement sensibles à ce composé. En ce qui concerne les autres organismes terrestres, on est parvenu à une valeur de 15 mg/m^3 pour la concentration sans effet en prenant la valeur limite pour l'hépatotoxicité chez la souris divisée par un coefficient d'application. En comparant cette valeur avec une estimation prudente de l'exposition on peut conclure que

dans le pays témoin, le DMF n'a vraisemblablement aucun effet nocif sur les organismes terrestres.

RESUMEN DE ORIENTACIÓN

Este CICAD sobre la *N*,*N*-dimetilformamida (DMF), preparado conjuntamente por la Dirección de Higiene del Medio del Ministerio de Salud del Canadá y la División de Evaluación de Productos Químicos Comerciales del Ministerio de Medio Ambiente del Canadá, se basó en la documentación preparada al mismo tiempo como parte del Programa de Sustancias Prioritarias en el marco de la *Ley Canadiense de Protección del Medio Ambiente* (CEPA). Las evaluaciones de sustancias prioritarias previstas en la CEPA tienen por objeto valorar los efectos potenciales para la salud humana de la exposición indirecta en el medio ambiente general, así como los efectos ecológicos. En este documento original no se abordó la exposición ocupacional. En este examen se analizaron los datos identificados hasta el final de septiembre de 1999 (efectos ecológicos) y febrero de 2000 (efectos en la salud humana). La información relativa al carácter del examen colegiado y la disponibilidad del documento original figura en el apéndice 1. También se consultaron otros exámenes, entre ellos el del IARC (1999) y el del BUA (1994). La información sobre el examen colegiado de este CICAD aparece en el apéndice 2. Este CICAD se aprobó como evaluación internacional en una reunión de la Junta de Evaluación Final celebrada en Helsinki (Finlandia) del 26 al 29 de junio de 2000. La lista de participantes en esta reunión figura en el apéndice 3. La Ficha internacional de seguridad química (ICSC 0457) para la *N*,*N*-dimetilformamida, preparada por el Programa Internacional de Seguridad de las Sustancias Químicas (IPCS, 1999), también se reproduce en este documento.

La *N*,*N*-dimetilformamida (CAS Nº 68-12-2) es un disolvente orgánico que se produce en grandes cantidades en todo el mundo. Se utiliza en la industria química como disolvente, intermediario y aditivo. Es un líquido incoloro con un ligero olor a amina. Es completamente miscible con el agua y la mayoría de los disolventes orgánicos y su presión de vapor es relativamente baja.

Cuando se libera en el aire, la mayor parte de las emisiones de *N*,*N*-dimetilformamida se mantienen en este compartimento, donde se degrada por reacción química con radicales hidroxilo. Las emisiones indirectas de *N*,*N*-dimetilformamida al aire, por ejemplo por desplazamiento desde otros compartimentos del medio ambiente, desempeñan sólo una pequeña función en el mantenimiento de los niveles de *N*,*N*-dimetilformamida en la atmósfera. Se estima que la fotooxidación de la *N*,*N*-dimetilformamida en el aire dura unos días. Sin embargo, parte de la *N*,*N*-dimetilformamida atmosférica puede alcanzar los medios acuático y terrestre, posiblemente con la lluvia. Cuando se libera *N*,*N*-dimetilformamida en el agua, se degrada allí y no pasa a otros compartimentos. Cuando se libera al suelo, la mayor parte de

la *N,N*-dimetilformamida se mantiene allí - posiblemente en el agua intersticial del suelo - hasta que se degrada por reacción biológica y química. Se supone que las emisiones al agua o al suelo van seguidas de una biodegradación relativamente rápida (semivida de 18-36 h). Si la *N,N*-dimetilformamida alcanza el agua freática, su degradación anaerobia será lenta. Las pautas de uso de la *N,N*-dimetilformamida hacen suponer que la exposición de la población general es probablemente muy baja.

Habida cuenta de que en el país de muestra la mayor parte de la *N,N*-dimetilformamida parece que se libera al aire y teniendo cuenta su destino en el medio ambiente, se supone que la biota está expuesta fundamentalmente a la *N,N*-dimetilformamida del aire; la exposición a la presente en las aguas superficiales, el suelo o los organismos bentónicos se supone que es escasa. Sobre esta base y debido a su baja toxicidad para una gran variedad de organismos acuáticos y del suelo, la caracterización del riesgo ambiental se concentra en los organismos terrestres expuestos directamente a la *N,N*-dimetilformamida del aire ambiente.

La *N,N*-dimetilformamida se absorbe fácilmente tras la exposición oral, cutánea o por inhalación. Después de la absorción, la *N,N*-dimetilformamida se distribuye de manera uniforme, se metaboliza sobre todo en el hígado y se excreta con relativa rapidez como metabolitos en la orina. En la vía principal interviene la hidroxilación de los grupos metilo, produciendo *N*-(hidroximetil)-*N*-metilformamida, que es el principal intermediario urinario en las personas y en los animales. La *N*-(hidroximetil)-*N*-metilformamida se puede descomponer a su vez para formar *N*-metilformamida. Luego, la oxidación enzimática del *N*-metilo de la *N*-metilformamida puede dar lugar a *N*-(hidroximetil)formamida, que a continuación se degrada a formamida. Una vía alternativa para el metabolismo de la *N*-metilformamida es la oxidación del grupo formilo, produciendo *N*-acetil-*S*-(*N*-metilcarbamoil)-cisteína, que ha sido identificado como un metabolito urinario en los roedores y en las personas. En esta vía se forma un intermediario reactivo, cuya estructura aún no se ha determinado (posiblemente metilisocianato); aunque no se han encontrado pruebas experimentales directas que lo respalden, parece que este intermediario es el metabolito supuestamente tóxico. Los datos disponibles indican que la proporción de *N,N*-dimetilformamida que se puede metabolizar por la vía supuestamente tóxica es mayor en las personas que en los animales de experimentación. Se ha detectado una interacción metabólica entre la *N,N*-dimetilformamida y el alcohol, lo cual, aunque no se conoce del todo, se puede deber, al menos en parte, a que inhibe la alcohol deshidrogenasa.

Coincidiendo con los resultados de los estudios en animales de experimentación, los datos disponibles de informes de casos y de estudios de muestras representativas de poblaciones expuestas ocupacionalmente indican que en las personas es el hígado el órgano destinatario de la toxicidad de la *N,N*-dimetilformamida. El perfil de los efectos está en consonancia con el observado en los animales de experimentación, habiéndose detectado trastornos gastrointestinales, intolerancia al alcohol, aumento de las enzimas hepáticas en el suero (aspartato aminotransferasa, alanina aminotransferasa, (-glutamil transpeptidasa y fosfatasa alcalina) y efectos histopatológicos y cambios ultraestructurales (necrosis hepatocelular, agrandamiento de las células de Kupffer, esteatosis microvesicular, lisosomas complejos, mitocondrías pleomórficas y cambios en la grasa con lipogranulomas ocasionales).

Teniendo en cuenta los limitados datos disponibles, no hay pruebas sistemáticas convincentes de un aumento del número de tumores en los lugares asociados con la exposición a la *N,N*-dimetilformamida en el entorno ocupacional. Las notificaciones de casos de cáncer testicular no se han confirmado en un estudio de cohortes y de casos y testigos. No se ha observado un aumento constante de tumores en otros lugares asociados con la exposición a la *N,N*-dimetilformamida.

Hay también pocas pruebas sistemáticas convincentes de genotoxicidad en las poblaciones expuestas ocupacionalmente a la *N,N*-dimetilformamida, con resultados desiguales en los estudios disponibles sobre trabajadores expuestos (a la *N,N*-dimetilformamida y a otros compuestos). La pauta de las observaciones no es coherente con las variaciones de la exposición en los diversos estudios. Sin embargo, a la vista de la relación dosis-respuesta positiva observada en el único estudio en el cual se investigó, convendría estudiar más este aspecto, aunque los datos disponibles sobre genotoxicidad en sistemas experimentales son abrumadoramente negativos.

La *N,N*-dimetilformamida tiene una toxicidad aguda baja y una actividad irritante ocular y cutánea entre ligera y moderada. No se identificaron datos relativos a su potencial de sensibilización. En estudios de toxicidad aguda y de dosis repetidas, la *N,N*-dimetilformamida ha sido siempre hepatotóxica, induciendo efectos en el hígado a las concentraciones o dosis más bajas. El perfil de los efectos incluye alteraciones en las enzimas hepáticas características de la toxicidad, aumento de peso del hígado, cambios histopatológicos de degeneración progresiva y a la larga muerte celular, así como aumento de las enzimas hepáticas en el suero. Tras la exposición por inhalación y por vía oral se ha observado una relación dosis-respuesta para estos efectos en ratas y ratones. Se ha detectado una variación de la sensibilidad entre especies para estos efectos, siendo el orden de sensibilidad ratones > ratas > monos.

Aunque la base datos para la carcinogenicidad se limita a dos biovaloraciones debidamente realizadas en ratas y ratones, no se ha registrado un aumento de la incidencia de tumores tras la exposición por inhalación crónica a la *N*,*N*-dimetilformamida. El valor probatorio para la genotoxicidad es totalmente negativo, basándose en una investigación amplia mediante valoraciones *in vitro*, en particular para la mutación genética, y en una base de datos más limitada *in vivo*.

En estudios con animales de laboratorio, tras la exposición tanto por inhalación como por vía oral la *N*,*N*-dimetilformamida indujo efectos reproductivos adversos sólo a concentraciones superiores a las asociadas con los efectos adversos en el hígado. Del mismo modo, en estudios fundamentalmente recientes sobre el desarrollo realizados y notificados de manera adecuada, se han observado sistemáticamente efectos citotóxicos y teratogénicos sólo a concentraciones o dosis con toxicidad materna.

Los estudios disponibles no son suficientes como base para la evaluación de los efectos neurológicos e inmunológicos de la *N*,*N*-dimetilformamida.

Este CICAD y la caracterización del riesgo en la muestra se concentran fundamentalmente en los efectos de la exposición indirecta en el medio ambiente general.

El aire en la proximidad de fuentes puntuales parece ser el principal origen potencial de exposición de la población general a la *N*,*N*-dimetilformamida. Sobre la base de los resultados de los estudios epidemiológicos de trabajadores expuestos y de los datos justificativos de una base de datos relativamente amplia de investigaciones en animales de experimentación, el hígado es el principal órgano destinatario de la toxicidad de la *N*,*N*-dimetilformamida. Se ha obtenido una concentración tolerable de 0,03 ppm (0,1 mg/m^3), teniendo en cuenta el aumento de las enzimas hepáticas en el suero.

No se han identificado datos sobre la toxicidad de la *N*,*N*-dimetilformamida para las plantas vasculares terrestres. Las concentraciones con efecto para los indicadores de una posible sensibilidad de los árboles, los arbustos y otras plantas son altas; por consiguiente, es poco probable que las plantas terrestres sean particularmente sensibles a la *N*,*N*-dimetilformamida. Para otros organismos terrestres, se ha estimado un valor sin efectos de 15 mg/m^3, basado en un valor critico de la toxicidad para la toxicidad hepática en ratones dividido por un factor de aplicación. La comparación de este resultado con un valor de exposición estimada prudente indica que es poco probable que la *N*,*N*-dimetilformamida provoque efectos adversos en los organismos terrestres del país de muestra.

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Exhibit 15

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CERTIFICATE OF ANALYSIS

COMMODITY:N,N-DIMETHYL FORMAMIDE

BATCH NO.:20101026

THE RESULTS OF INSPECTION ARE AS FOLLOWS:

ITEM	SPECIFICATION	RESULTS
Pt-Co chrominance	≤10	5
Distillation test 0° C 101325Pa 151-155° C distillate volume	≥98.5	98.6
dimethylamine ppm	≤15	1
Formic acid ppm	≤25	2
PH:25° C 20%Aqueous solution	7.0-7.5	7.05
Electric Conductivity: 25° C 20%Aqueous solution, us	≤10	1.1
Moisture ppm	≤500	122
Fe ppm	≤0.05	0.002
Refractivity: $n_d^{25^\circ c}$	1.4270-1.4290	1.4286
Methanel ppm	≤20	2
Heavy compoment (dimethyl Acetamide) ppm	≤500	43
DMF %	≥99.9	99.98

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Exhibit 17

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E-mail: trade@chinaorganicchem.com sale@chinaorganicchem.com

CERTIFICATE OF ANALYSIS

DATE:NOV.25,2012
LOT NO.:201209-207-80

Triethylamine Analysis

Property		Specifications	Results
Triethylamine,	wt%	99.5 Min	99.9
Water,	wt%	0.1 Max	0.02
Monoethylamine,	wt%	0.1Max	0.01
Diethylamine,	wt%	0.1Max	0.01
Ethanol,	wt%	0.1Max	N.D
Color ,	APHA	15 Max	10

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ZHEJIANG JIANYE CHEMICAL CO., LTD.

Sign: 郑丰平

Exhibit 18

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DIMETHYLFORMAMIDE: PURIFICATION, TESTS FOR PURITY AND PHYSICAL PROPERTIES

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Analytical Chemistry Division

Commission on Electroanalytical Chemistry

DIMETHYLFORMAMIDE: PURIFICATION, TESTS FOR PURITY AND PHYSICAL PROPERTIES[+]

N,N'-Dimethylformamide (DMF) is a good solvent for organic and to a lesser extent inorganic compounds. It is, together with dimethylsulfoxide and acetonitrile, one of the most widely used of the so-called dipolar aprotic solvents. Owing to its fairly high dielectric constant, it is a moderately dissociating solvent for electrolytes. Acid-base reactions as well as thermodynamic properties of electrolyte solutions have been studied by many authors. Contrary to the N-methylamides it is a typically weakly associated solvent, as seen (Ref. 1) from dielectric studies (the Kirkwood g factor is about one at all temperatures).

Owing to its electron-donor character, DMF reacts with many acids. For example, Gutmann's donicity number (Ref. 2) is 27. Its polarographic range is quite large, e.g., 3.5 V at the dropping mercury electrode with 0.1 M Bu_4NClO_4 as supporting electrolyte (Ref. 3). It is therefore widely used as a solvent for electrochemical reactions, especially reductions.

Pure DMF is colorless and, at room temperature, odorless. It is subject to thermal as well as photochemical degradation. In presence of water, DMF is slowly hydrolyzed according to the equation:

$$HCON(CH_3)_2 + H_2O \longrightarrow HCOOH + (CH_3)_2NH$$

Formic acid and dimethylamine are thus predominant impurities in DMF and determine the odor of the impure solvent. They are weakly acidic and weakly basic respectively; therefore, partial ionization does occur:

$$HCOOH + (CH_3)_2NH \rightleftharpoons HCOO^- + (CH_3)_2NH_2^+$$

and results in a buffered solution (pH 11) with an increase in the conductivity of the solvent.

Thermal degradation produces dimethylamine and carbon monoxide. Hydrogen (Ref. 4) and hydrogen cyanide (Ref. 5) have been identified among the products of the photochemical degradation of the solvent.

Strongly basic media are difficult to obtain in DMF; there is, to our knowledge, no substance behaving as a strong base in DMF. If autoprotolysis of the medium actually occurs, the anion of the solvent must be very unstable (Ref. 6). It has been claimed (Ref. 7 and 8) that the autoprotolysis constant is smaller than 10^{-25} but no definite value has yet been proposed.

Attention must be paid to the fact that DMF has toxic effects, particularly on the liver and kidneys; the threshold value for air has been fixed (Ref. 9) at 30 mg/m^3.

PURIFICATION OF DIMETHYLFORMAMIDE

Good quality DMF is commercially available. As noted by Vaughn (Ref. 10), spectrograde solvent is not always suitable for all purposes. As a consequence of hydrolysis, the residual water content of commercial DMF is frequently low (0.1%). Many procedures have been proposed and used for the purification of the solvent. Four types of successive operation can be distinguished: treatment with a drying agent, neutralization of basic or acidic impurities, careful distillation, and elimination of gaseous impurities.

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1. Preparing water-free solvent. Although the boiling point of water is far from that of DMF it is not possible to obtain a dry solvent by distillation only.

One of the first methods proposed for preliminary drying (Ref. 11) was azeotropic distillation with about 10% by volume of dry benzene; the benzene-water azeotrope is removed by distillation at atmospheric pressure. To prevent decomposition the temperature is maintained below 80°C. Alternatively, molecular sieves can be used. The solvent is kept in contact for periods ranging from 1 to 4 days with 4 Å (Ref. 12-15) or 5 Å (Ref. 16) sieves which are removed and replaced from time to time. Ritchie (Ref. 17) recommends the use of Linde AW-500 molecular sieves in 1/16-inch pellets. Studying drying efficiency, he finds that the water content is less than 18 ppm after 27 hours. Molecular sieves can be dried before use by heating in a quartz tube under a stream of argon at 375°C for 24 h (Ref. 18). Finally, a procedure which uses chromatographic purification through alumina has been described by Moe (Ref. 13) in some detail. "A column approximately 100 cm long and 5 cm wide will contain 1 kg of alumina, sufficient for the purification of about 10 l of DMF". After bubbling of pure nitrogen for several hours the DMF thus obtained is thought to be convenient for polarographic use.

In our opinion these three types of operation can be considered only as a first step in drying the solvent, and mild chemical drying agents must also be used. These range from anhydrous BaO (Ref. 11 and 19) to $MgSO_4$ (Ref. 20), Na_2CO_3 (Ref. 6 and 20), or $CuSO_4$ (Ref. 21). Surprisingly good samples of DMF can be obtained using storage of solvent over these chemicals for at least 24 h. It has been recommended that the drying agent be changed at least twice and the container shaken, if not continuously, at least from time to time. It also has been recommended that such an operation is performed in a cold, dark room. As far as Na_2SO_4 or Na_2CO_3 are concerned, the resulting solvents are of about the same quality (Ref. 20). Little or no degradation of the solvent (as estimated through the concentration of dimethylamine) results from such treatment (Ref. 11).

Some of the more common drying agents react with the solvent itself to produce significant amounts of acidic or basic impurities. BaO, cited previously, belongs to this category if it is used at temperatures above 30°C (Ref. 11). Other reagents are potassium hydroxide, calcium hydride (Ref. 5 and 22) and phosphorus pentoxide (Ref. 17, 23 and 24). P_2O_5 is the most frequently used, CaH_2 is probably the most efficient. Prue and Sherrington (Ref. 23) have shaken DMF for three days with P_2O_5, adding each morning about 10 g of fresh reagent. Recently, drying of amides using Vitrid, sodium bis(methoxy-2-ethoxy)aluminohydride, has been recommended (Ref. 25). In DMF it allows attainment of very basic media (pH 30). However, distillation of the solvent from the mixture obtained has not been attempted and is probably very hazardous. Whatever the method used, it is important to proceed with these operations in a dark room or apparatus to prevent any photochemical degradation.

2. Neutralization. Depending on the drying agent used, it has been recommended that the basic or acidic impurities produced are neutralised, either by shaking with picric acid (Ref. 20) or KOH pellets (Ref. 24). This last treatment is particularly recommended after drying over P_2O_5 which generates formic acid. Such neutralization can be done either before or after a first distillation.

3. Distillation. The drying process can be further carried out during this operation. The DMF is refluxed and distilled from P_2O_5 or CaH_2. However, owing to a degradation process increased by heating, it is preferable first to decant the solvent and transfer it under dry nitrogen, and then to distil it at reduced pressure.

The quality of the final product is greatly affected by the care with which the distillation is carried out. It seems to be important to work under vacuum, with a darkened column, or in a pure nitrogen or argon atmosphere. As a rule, the temperature must be kept under 60°C; heating must be gentle and overheating avoided. Distillation in daylight results in the production of hydrogen cyanide (Ref. 5), particularly in the presence of CaH_2. No traces of HCN are detected if the operations are conducted in the dark.

Types of distillation apparatus currently described in the literature do not seem to be very efficient. It is not surprising to note that the best quality DMF, if conductivity is accepted as a test of purity, has been obtained by Brummer (Ref. 12), who used only molecular sieves as drying agents, but carried out the distillation in a slow current of dry nitrogen at low pressure (2 torr) and an efficient column (1 meter packed with Fenske helices). The use of a long column (60 cm at least) with good packing and reflux is recommended. For example, Tanaka (Ref. 21) distilled DMF which had been dried over anhydrous $CuSO_4$ at a pressure of 5 torr through an adiabatic fractional distillation column which was 1.3 cm in diameter, 120 cm in length and packed with helipack coils. Dry nitrogen was passed through the apparatus during the distillation; 60% of the distillate was collected. The conductivity was lower than $1 \times 10^{-7}\ \Omega^{-1}\ cm^{-1}$ (25°C). Boiling temperatures at various pressures are given in Table 1.

TABLE 1. Recommended values for physical constants of DMF at 25°C and 1 atm (except where noted otherwise)

Boiling temperature	T_B	152.3°C (Ref. 47) 79°C at 61-62 torr (Ref. 37) 55-56°C at 25-26 torr (Ref. 40) 34°C at 2-3 torr (Ref. 15)
Melting temperature	T_M	-61°C
Refractive index (Ref. 44)	n_D^{25}	1.42689
Dielectric constant	D	37.0
Surface tension (Ref. 45)	σ	37.1 dyne/cm
Viscosity (Ref. 23)	η	0.00796 poise
Density	ρ	0.9440 g cm^{-3}
Molal volume	V	77.39 cm^3
Heat capacity at constant pressure (Ref. 44)	C_p	37.4 cal/mol
Cubic expansion coefficient	α_P	1.00×10^{-3} K^{-1} *
Adiabatic compressibility coefficient (Ref. 44)	β_S	6.1×10^{-5} atm^{-1}
Isothermal compressibility coefficient	β_T	6.3×10^{-5} atm^{-1} *

*Calculated from data in Ref. (12)

4. Elimination of gaseous impurities. A flow of pure dry nitrogen or argon is passed through the solvent for several hours, in order to eliminate oxygen, carbon monoxide and carbon dioxide. Such a solvent can then be used for polarographic purposes. A more complete deaeration can be achieved using a vacuum line.

5. Conclusions and recommendations. As various authors used different starting materials, it is difficult to compare the efficiency of the various methods of purification. Comparison between different ways of treating the same batch of solvent can be found to our know ledge in only two papers (Ref. 11 and 20). Thomas and Rochow (Ref. 11) always used first azeotropic distillation with benzene and compared subsequent treatment with $MgSO_4$, BaO, alumina and triphenylchlorosilane, followed in each case by distillation. Comparisons were made in terms of specific conductance and water content. Barium oxide as well as alumina treatment meet rather well these two criteria and do not have any side effects, such as producing dimethylamine or HCN. Juillard (Ref. 20) compared drying with Na_2CO_3, Na_2SO_4 or molecular sieves with azeotropic distillation with benzene and distillation over P_2O_5. As far as conductivity and water content are concerned, the different batches of solvent thus obtained were of about the same quality, except that P_2O_5 has the disadvantage of promoting degradation of the solvent and thus of decreasing the efficiency of the distillation; therefore the use of P_2O_5 is not recommended. As a confirmation it can be noted that authors using P_2O_5 or CaH_2 as drying agents did not obtain purer solvents than those who employed BaO or Na_2CO_3 or even only molecular sieves.

It is therefore recommended that use is made first either of azeotropic distillation with benzene, as suggested by Thomas (Ref. 11), or of treatment with molecular sieves, as suggested by Ritchie (Ref. 17), and that the resulting DMF is then shaken with Na_2CO_3 or, better, with BaO for 1 or 2 days. After decantation the DMF is distilled twice under nitrogen (pressure <15 torr) using a 1-m column. All these operations must be carried out in the dark. After deaeration the solvent is stored under nitrogen and used as soon as possible.

TESTS FOR PURITY

Owing to its various modes of degradation (hydrolysis, thermal and photochemical decomposition) the principal impurities found in DMF are: dimethylamine, formic acid, hydrogen cyanide, carbon dioxide and carbon monoxide. To this list must be added: water, oxygen, which is quite soluble, and impurities resulting from the purification process.

Conductivity. As stressed earlier, hydrolysis as well as decomposition results in ionic impurities: dimethylammonium formate, carbonate or cyanide. Thus, the conductivity of the solvent is a very good test of its purity.

Experimental conductivities recorded in DMF are always higher than those reported for other aprotic solvents such as ketones or nitriles. According to a rough estimate, the theoretical conductivity of the solvent should be below 10^{-13} $\Omega^{-1}cm^{-1}$. In fact, conductivities obtained by the most careful workers are scarcely ever less than 10^{-7}. The best values have been reported, to our knowledge, by Brummer (Ref. 12) who used for conductometric studies a solvent having conductivities varying from 2×10^{-8} to 5×10^{-8} Ω^{-1} cm^{-1}. Values below 5×10^{-7} have been reported by numerous authors and any batch of DMF which is more conducting can be considered to be impure.

Water. Water can be titrated by the Karl Fischer (K-F) reagent. Kanatharan (Ref. 22) recommends that the titration is conducted slowly, since K-F reagent reacts only slowly with small amounts of water.

Usual procedures do not allow the determination of less than 10 ppm of water. According to Muroi (Ref. 26) it is possible to titrate as little as 0.2 ppm by increasing the sharpness of the end point, using the following procedure: "Add a 10-30 ml sample to 25 ml MeOH containing 8% of a pyridine-SO_2 solution (320 g SO_2/l pyridine) and titrate potentiometrically with K-F reagent having a titre of 0.1-0.5 mg H_2O/ml". The use of DMF as a solvent for K-F reagent also has been advocated (Ref. 27).

Prue (Ref. 23) has titrated water in DMF using triphenylsilyl chloride, from which, according to Thomas (Ref. 11), hydrogen chloride is liberated quantitatively by water (amines or acids are thought to interfere); the HCl content is then estimated from the conductivity of the solution.

It is quite easy to prepare a solvent which contains less than 50 ppm of water. Very low concentrations (< 5ppm) are more difficult to attain. The best value, less than 3 ppm, has been reported by Libbey and Stock (Ref. 28).

Dimethylamine. Colorimetric methods have been used by some authors. In our opinion, as long as the autoprotolysis constant of the solvent is not known, it is not possible to say exactly what is basic and what is acidic in DMF. Kolthoff (Ref. 24) has used p-nitrophenol in the colorimetric determination of total basicity, but specific determinations would be preferable.

Thomas and Rochow (Ref. 11) have based the determination of the amine content on the fact that dimethylamine forms with 1-fluoro-2,4-dinitrobenzene a complex which absorbs in the visible spectrum at 3812 Å. Solvent prepared by Chang and Criss (Ref. 29) was found to contain less than 1 ppm of dimethylamine using this method.

Another spectrophotometric method which allows the determination of the dimethylamine content down to 2 ppm with an error of $\pm 10\%$ has been proposed by Pribyl (Ref. 30); dimethyldithiocarbamate, which absorbs at 445 nm, is formed by adding CS_2 and $Cu(AcO)_2$ to an EtOH-pyridine mixture.

Chromatography was thought by Butler (Ref. 18) not to be a reliable means of establishing the organic impurity content of the solvent since DMF can decompose or hydrolyze at high temperatures. Nevertheless, careful studies of the proper experimental conditions have been undertaken (Ref. 31 and 32). In the paper by Filippov (Ref. 32) it is shown that dimethylamine can be determined in DMF at levels as low as 1 ppm using tetrahydroxyethylenediamine as a stationary phase, polysorb-1 as a solid support and a column temperature of 75°C.

Dimethylamine is not electroactive with mercury but can give coordination compounds with cations which will affect the course of electrochemical reductions.

Formic Acid. In contrast to dimethylamine, formic acid is electroactive. Kanatharan and Spritzer (Ref. 22) have attributed to formic acid two peaks, one cathodic, the other anodic, which appear in cyclic voltammograms of aqueous dimethylformamide. Alternating current polarography (Ref. 33), and, better, pulse polarography, can be used to estimate the formic acid content.

Formic acid can also be determined by titration with a base. Potentiometric titration is preferred since it allows determination of the dimethylammonium formate content as well. Megliskii (Ref. 34) has titrated potentiometrically formic acid, dimethylamine and dimethylammonium formate in DMF using two solutions: 0.1 M $HClO_4$ and 0.1 M KOH, both in alcohol. Such a method is suitable only for concentrations of the order of at least 100 ppm.

Hydrogen Cyanide. Trisler et al. (Ref. 5) reported the presence of HCN in DMF distilled over CaH_2 in natural light. Concentrations ranged from 10^{-5} to 10^{-3} M. Spectrophotometric titration can be carried out with 4-nitrobenzil, which reacts with cyanide ion to form a deep violet ion.

Oxygen. Oxygen is rather soluble in DMF. A study of oxygen solubility in relation to the oxygen content of the gas phase has been made by James (Ref. 35). When the gas phase was air and pure oxygen, the solubility was 2.2×10^{-3} and 3.1×10^{-3} M, respectively.

Oxygen is an electroactive impurity which interferes in polarography and other electrochemical processes. Two waves are observed (Ref. 36) with $E_{1/2} = -0.8$ and -2.8 V vs. SCE; the first corresponds to the reduction of oxygen to superoxide:

$$O_2 + e^- \longrightarrow O_2^{\cdot -}$$

and the second one to the reduction of superoxide to peroxide ion:

$$O_2^{\cdot -} + e^- \longrightarrow O_2^{=}$$

James (Ref. 35) has proposed two methods for the determination of the oxygen concentration; polarography and the Winkler method. Polarographic measurements are made at -1.2 V vs. SCE, in order to ensure that the measured diffusion current is not influenced by a polarographic maximum. A modified Winkler method allows concentrations as low as 10 ppm to be determined. It depends upon quantitative oxidation of iodide ion to iodine. Such a process is described in some detail (Ref. 35).

PHYSICAL PROPERTIES OF DIMETHYLFORMAMIDE

Numerical values of physical constants are highly dependent on the purity of the solvent. Consequently, important discrepancies are found in the literature. The present recommended values result from a careful examination of three aspects: accuracy of the measurements, consistency of the data of various authors at different temperatures, and purification of the solvent. Such a choice is subject to personal evaluation and it seems prudent to give also the other references.

Density. The density is probably a good criterion of the purity of the solvent. Contamination with water increases the density (Ref. 23). The following values of the density at 25°C have been found (Ref. 23,8,37,12): 0.9439, 0.9440_2, 0.9441_5 and 0.9442 g cm^{-3}, respectively. Old values greater than 0.9443 frequently found in tables are probably too high. New work by Kawaizumi and Zana (Ref. 38) seems to indicate that the density of the pure solvent is lower. These authors obtain values ranging from 0.94360 to 0.94368. It is our feeling that these data are more accurate than previous ones but such a low value ($\rho = 0.94364 \pm 0.00004$) must be confirmed by others before being accepted.

Values at various temperatures other than those appearing in Table 2 have been given by Gopal and Rizvi (Ref. 39). At 20°C Saphon (Ref. 40) has obtained $\rho = 0.94878$ g cm^{-3}, in good agreement with the value in Table 2.

TABLE 2. Recommended values for physical constants of DMF at various temperatures

		ρ g cm^{-3}	η poise	D
Temperature	20°C	0.9488	0.00845	38
	30°C	0.9394	0.00746	36.1
	40°C	0.9298	0.00664	34.4
	50°C	0.9202	0.00598	32.8
Reference		12	49	1

Viscosity. Other values can be found in References (29) and (41). Prue's data at 25°C are confirmed by measurements reported by Ames and Sears (Ref. 42).

Dielectric constant. Data given by Bass and Cole (Ref. 1) are preferred to previous results (Ref. 43) of Leader and Gormley (36.71 at 25°C). The value reported at 25°C is interpolated from measurements at various temperatures. Data of Saphon (Ref. 40) are in good agreement with the value reported in Table 2 at 20°C (D = 38.13).

Miscellaneous. Data at various temperatures concerning refractive index, surface tension and isothermal compressibility can be found in Refs. (44), (45) and (12), respectively. Other data concerning thermodynamic properties are reported in Refs. (39) and (44). Plots of vapor pressure, heat of vaporization, heat capacity, density, viscosity, surface tension and thermal conductivity for a large range of temperature have been drawn up by Gallant (Ref. 46). The solubilities of some sixty substances in DMF have been tabulated (Ref. 50). Organic reactions in or with DMF have been summarized (Ref. 51).

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Exhibit 20

Notice on the Results of the Report of the Preliminary Investigation on the Formation of Unknown Impurities Resulting from the Sodium Azide Quenching in Crude Irbesartan

Jinsheng LIN

To: Jucai GE, Tianpei HUANG, Wangwei CHEN, Wenquan ZHU, Wenbin CHEN, Mr. Li, Peng DONG, Lihong LIN, Yanfeng LIU, Peng WANG, Wenling ZHANG 07/27/2017 Detailed Information

Valsartan Impurity K.pdf (846 KB)

Ms. Ge:

According to the results of our telephone communication with the Technology Department of Chuannan Plant 1 today, due to the incomplete quenching of sodium azide caused by the separate treatment of irbesartan sodium azide wastewater, there is the frequent occurrence of muffled explosion in the production process, so the Technology Department carried out the technical improvement by which the sodium azide quenching takes place in the unstratified step in the crude irbesartan process. However, after the improvement, there is an unknown impurity of about 0.544% at 26 minutes in the crude irbesartan, and it is the largest impurity in the irbesartan crude product.

[redacted]

Min Li
ZHP-296
4/20/2021

Through the secondary mass spectrometry analysis, it can be inferred that the extra NO substituent is in the cyclic compound fragment, and it is very likely that it is an N-NO compound; it is similar to the N-nitrosodimethylamine that occurs in valsartan when quenched with sodium nitrite, and its structure is very toxic. Its possible formation route is shown as follows:

In order to further verify the structure of the impurity and its formation mechanism, we plan to simulate the quenching conditions and use the finished Irbesartan product to react with $NaNO_2$ and HCl to monitor the impurity produced by the reaction, and then separate it for NMR for final structural verification, while simultaneously carrying out the confirmation of the impurity by multi-stage MS.

If it is confirmed as the above speculated structure, then its toxicity will be very strong, and there will be an extremely high GMP risk. This is a common problem in the production and synthesis of sartan APIs. It is recommended to improve other quenching processes (such as NaClO) along with the optimization of the valsartan sodium azide quenching process.

I've also attached a patent of a 2013 sodium azide NaClO quenching method by Zhejiang Second Pharma Co., Ltd. they proposed that the use of $NaNO_2$ quenching will result in the formation of N-NO impurities. At the same time, they used ZHP's crude Valsartan in their LC-MS test and detected this impurity. This indicates that other companies have paid attention to the quality problem very early on. So leaders please pay attention to this issue.

Jinsheng LIN

CEMAT

2017/07/27

Exhibit 21

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY
CAMDEN VICINAGE

IN RE: VALSARTAN, LOSARTAN, AND IRBESARTAN PRODUCTS LIABILITY LITIGATION	MDL No. 2875 Honorable Robert B. Kugler, District Court Judge Honorable Karen M. Williams, Magistrate Judge Honorable Thomas Vanaskie (Ret.), Special Discovery Master

DECLARATION OF SETH A. GOLDBERG

I, Seth A. Goldberg, of full age, hereby declare as follows:

1. I am an attorney at law of the State of New Jersey, a member of good standing of the bar of this Court, a Partner with the law firm of Duane Morris LLP, and counsel to Defendants Zhejiang Huahai Pharmaceutical Co., Ltd. ("ZHP"), Prinston Pharmaceutical Inc. ("Prinston"), Solco Healthcare U.S. ("Solco"), and Huahai U.S. Inc. ("Huahai U.S.", and collectively with ZHP, Prinston, and Solco, "the ZHP Parties").

2. I make this Declaration based on personal knowledge and in support of the ZHP Parties' Cross Motion for Protective Order to Preclude the Production of the Custodial File of Baohua Chen.

3. A true and correct copy of the final transcript of Jucai Ge's deposition testimony on April 30, 2021 is attached to this Declaration as **Exhibit A**.

4. A true and correct copy of the final transcript of Lihong (Linda) Lin's deposition testimony on May 4, 2021 is attached to this Declaration as **Exhibit B**.

5. A true and correct copy of the final transcript of Min Li's deposition testimony on April 22, 2021 is attached to this Declaration as **Exhibit C**.

6. A true and correct copy of the final transcript of Eric Gu's deposition testimony on April 5, 2021 is attached to this Declaration as **Exhibit D**.

7. A true and correct copy of the final transcript of Hai Wang's deposition testimony on March 11, 2021 is attached to this Declaration as **Exhibit E**.

8. A true and correct copy of the final transcript of John Iozzia's deposition testimony on January 20, 2021 is attached to this Declaration as **Exhibit F**.

9. Attached to this Declaration as **Exhibit G** is a table listing the titles held by Baohua Chen in various public bodies.

10. Attached to this Declaration as **Exhibit H** is an index cataloging the volumes of the ZHP Parties' productions to date in this litigation.

11. Attached to this Declaration as **Exhibit I** is a list of the custodial files collected in connection with the ZHP Parties' productions in this litigation.

12. Attached to this Declaration as **Exhibit J** is a table illustrating the references to Baohua Chen in the exhibits introduced at the depositions of the ZHP Parties witnesses.

13. A true and correct copy of an English language translation of an e-mail dated July 27, 2017, authored by ZHP employee Jinsheng Lin (ZHP00190573) is attached to this Declaration as **Exhibit K**.

14. A true and correct copy of an e-mail dated April 23, 2021 from Plaintiffs' counsel to counsel for the ZHP Parties is attached to this Declaration as **Exhibit L**.

15. A true and correct copy of a declaration executed by Linhong (Linda) Lin, the Director of Regulatory Affairs of ZHP, is attached to this Declaration as **Exhibit M**.

16. A true and correct copy of a declaration executed by Yang Xueyu, a partner of Yu Zheng Law Firm, is attached to this Declaration as **Exhibit N**.

17. Attached to this Declaration as **Exhibit O** is a table comparing the volume of correspondence including Baohua Chen to the total production volume of the ZHP Parties to date in this litigation.

Executed on May 14, 2021.

Respectfully submitted,

/s/ Seth A. Goldberg

Seth A. Goldberg, Esq.
Lead Counsel and Liaison Counsel for Defendants

DUANE MORRIS LLP

Seth A. Goldberg, *Lead Counsel and Liaison Counsel for Defendants*
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Attorneys for Zhejiang Huahai Pharmaceutical Co, Ltd., Prinston Pharmaceutical Inc., and Solco Healthcare US, LLC

EXHIBIT K

Bulletin on the preliminary findings about produced unknown impurities in quenching sodium azide for the crude irbesartan

Lin Jinsheng July 27, 2017, 4:17 PM Detailed information

To: Ge Jucai, Huang Tianpei, Chen Wangwei, Zhu Wenquan, Chen Wenbin, Li Zong, Dong Peng, Lin Lihong, Liu Yanfeng, Wang Peng, Zhang Wenling

[icon] Valsartan Impurities K.pdf (846 KB)

General Manager Ge:

According to the results in our telephone communication with the Chuannan (Southern Sichuan)-Technical Department I today, because the separate treatment of sodium azide wastewater of irbesartan resulted in incomplete quenching of sodium azide, resulting in frequent depressed blast in the production process, thus, the technical department carried out technical transformation to quench sodium azide in the no stratification process of the crude irbesartan process, however, after the transformation, 0.544% of unknown impurities are produced in the crude irbesartan at 26 min, and it is the biggest impurity in the crude irbesartan..

[redacted]

Through the secondary mass spectrometry analysis, it can be inferred that the additional NO substituent is in the cyclic compound fragment part, and it is probably that it is the N-NO compound, similar to the N- nitrosodimethylamine group produced by the quenching of valsartan with sodium nitrite, its structure is very toxic, and its possible production pathways are as follows:

In order to further confirm the structure of the impurity and the principle of its generation, we plan to simulate the quenching conditions to react NaNO2 and HCI with the finished product of irbesartan, to monitor the impurities produced by the reaction, and then separate them for NMR for final structural confirmation, simultaneously, carry out the confirmation of impurity by multi-stage mass spectrometry.

If it is confirmed as the above speculated structure, its toxicity will be very strong, and GMP risk is great. This is a common problem in the production and synthesis of sartan API. It is recommended to improve to other quenching method, such as NaCIO, in addition to optimize the quenching process for sodium azide in valsartan.

Attached is a patent method for quenching sodium azide with NaCIO by Xinsaike Pharmaceutical in 2013. they proposed that the use of NaNO2 quenching will produce N-NO impurities, in the meanwhile, our Huahai crude valsartan was detected by LC-MS. The impurity was indeed found, indicating that other companies have paid attention to this quality issue a long time ago. Leaders are also requested to pay attention to it.

Lin Jinsheng
CEMAT
July 27, 2017

Exhibit 24

Masonic Cancer Center
University of Minnesota
Comprehensive Cancer Center designated by the National Cancer Institute

2231 6th Street SE
Room 2-148 CCRB
Minneapolis, MN 55455
Phone: (612) 624-7607
Fax: (612) 624-3869
hecht002@umn.edu

July 6, 2021

Adam M. Slater
Mazie Slater Katz & Freeman
103 Eisenhower Parkway
Roseland, New Jersey 07068

Dear Mr. Slater:

At your request I have reviewed scientific literature, corporate documents, deposition testimony, and regulatory documents and standards, as set forth in this report and the attached Exhibits, and applied my education, training, and knowledge to provide opinions related to the contamination of valsartan manufactured by API manufacturers including ZHP, Hetero, Mylan, and Aurobindo with nitrosamines, and in particular NDMA and NDEA (the "contaminated valsartan"), and then incorporated into finished dose form by the same manufacturers, as well as finished dose manufacturers Teva and Torrent, who purchased the API from the API manufacturers (Teva from ZHP and Mylan, Torrent from ZHP) and incorporated the contaminated valsartan into their finished doses.

As set forth in detail herein, it is my opinion that the NDMA and NDEA levels found in the contaminated valsartan were completely avoidable and therefore are and were unreasonably dangerous, causing an increased risk for the development of cancer for those people ingesting the contaminated valsartan. All opinions set forth herein are held to a reasonable degree of scientific certainty, and have been formed based upon application of scientifically validated methodologies that I utilize in my own scientific work. My background and credentials are set forth in my curriculum vitae, which is attached hereto as Exhibit 1. The list of documents reviewed as part of my analysis is attached hereto as Exhibit 2. The list of scientific literature references specifically relied on for my opinions is attached hereto as Exhibit 3, with the caveat that the scientific literature relevant to the issues addressed is vast, and my familiarity with that literature certainly informs my knowledge in this field, as does all of my experience, even if not specifically listed.

Stephen S. Hecht

Stephen S. Hecht, Ph.D.
Wallin Professor of Cancer Prevention
American Cancer Society Professor
American Chemical Society Fellow

NCI Designated Comprehensive Cancer Center

I. Professional Background

I will first provide an overview of my professional background. I received my B.S. degree in chemistry (with honors) from Duke University in 1964 and my Ph.D. in organic chemistry from the Massachusetts Institute of Technology (MIT) in 1968. From 1968-69, I held a postdoctoral fellowship position at MIT in the laboratory of Professor Klaus Biemann, a pioneer in the application of mass spectrometry to organic chemical analysis. My research helped lay the groundwork for analysis of samples to be returned by NASA astronauts from the moon, and analyzed for trace organic molecules using mass spectrometry. I was an assistant professor of chemistry at Haverford College from 1969-1971, and a National Research Council Fellow at the U.S. Department of Agriculture from 1971-1973, carrying out research on practical applications for utilizing excess animal fat. My research career on nitrosamines began when I joined the American Health Foundation in 1973, initially as Head of the Section of Organic Chemistry in the Division of Environmental Carcinogenesis (1973-1980), then as Head of the Division of Chemical Carcinogenesis (1980-1996), and concurrently as Director of Research for the Foundation (1987-1996). The American Health Foundation was a private research institute founded by the eminent epidemiologist Ernst L. Wynder. I will discuss my research in more detail later, but I note here that I have carried out research related to nitrosamines continually since 1973. I have been continually funded for this research by the U.S. National Cancer Institute since 1975. Among a number of highly important contributions to the nitrosamine research field, my colleagues and I were the first to characterize "tobacco-specific nitrosamines" in tobacco products. These nitrosamines, among which are the powerful cancer causing agents NNK (Nicotine-derived nitrosamine ketone) and NNN (*N*-Nitrosonornicotine), considered "carcinogenic to humans" by the International Agency for Research on Cancer, are widely viewed as some of the main cancer causing agents in tobacco products. Our research paper in the 1978 Journal of the National Cancer Institute, describing these compounds, has been cited by the American Association for Cancer Research as a "Landmark in Cancer Research." In 1996, I relocated to the University of Minnesota where I hold my current position as Wallin Professor of Cancer Prevention, a "Land Grant Endowed Chair" in cancer prevention research. My academic appointment is in the Department of Laboratory Medicine and Pathology, in the University of Minnesota Medical School. I am also a member of the Medicinal Chemistry and Pharmacology graduate programs. From 1998-2014, I was the founding Head of the Carcinogenesis and Chemoprevention Program of the Masonic Cancer Center, University of Minnesota, a National Cancer Institute designated Comprehensive Cancer Center. I currently lead a research group of 10-15 scientists with B.S., M.S., or Ph.D. degrees in the chemical and biological sciences. Our research, which focuses on mechanisms and prevention of cancer induced by tobacco products and environmental agents, is fully funded by grants from the U.S. National Cancer Institute and the National Institute of Environmental Health Sciences. I am the principal investigator of three R01 grants and a program project (P01) grant, from the National Cancer Institute and co-investigator on a number of other grant and cooperative agreement awards from the National Institutes of Health and the Food

and Drug Administration. I have been awarded a Merit Award (10 years of funding) and an Outstanding Investigator Grant (14 years of funding) from the National Cancer Institute. My research has been recognized by a number of prestigious awards, including election as a Fellow of the American Association for the Advancement of Science, a Fellow of the American Chemical Society, and a Research Professor of the American Cancer Society. To the best of my knowledge, I am the only scientist who has ever held the latter two awards from the American Chemical Society and the American Cancer Society simultaneously. I received the American Association for Cancer Research/Cancer Research and Prevention Foundation Award for Excellence in Cancer Prevention Research in 2006 and the Founders Award from the Division of Chemical Toxicology, American Chemical Society, in 2009. I received the Joseph Cullen Award from the American Society of Preventive Oncology in 2012. I received the William Cahan Distinguished Professor Award from the Flight Attendant Medical Research Institute in 2002 and the Alton Ochsner Award Relating Smoking and Health in 2001. I received the Minnesota Award from the Minnesota Section of the American Chemical Society in 2017. I am a member of the Academy for Excellence in Health Research and the Academy for Excellence in Team Science at the University of Minnesota. My publication entitled "Tobacco Smoke Carcinogens and Lung Cancer", published in the Journal of the National Cancer Institute in 1999, can be found on the University of Minnesota Medical School "Wall of Scholarship" because it was cited more than 1,000 times in the peer-reviewed literature. I served as Editor-in-Chief of the American Chemical Society journal *Chemical Research in Toxicology* from 2013-2017 and as an Associate Editor of the *Journal of Medicinal Chemistry* from 2004-2012.

Respected researchers are invited by the National Institutes of Health as well as private foundations to serve on peer review groups for evaluation of research proposals submitted by scientists from the U.S. and abroad. I was chair of the "Chemo-Dietary Prevention Study Section" of the NIH from 2006-2009, and served on the "Chemical Pathology Study Section" from 1981-1985. I was on the Board of Scientific Counselors of the National Cancer Institute from 2001-2004. I served on the "Carcinogenesis, Nutrition, and the Environment Study Section" of the American Cancer Society from 1998-2001, and as its Chair in 2001. I served on the American Cancer Society "Council for Extramural Grants" from 2010-2014. I served on the "Grants Review Panel" for the American Institute for Cancer Research from 1984-1987. I currently serve, since 2011, on the National Cancer Institute "PREVENT Study Section." I continue to be in demand as an ad hoc member of multiple other peer review panels.

Numerous other service activities to the scientific research community are listed in my Curriculum Vitae. I note some of the more important ones here. I have served on multiple writing groups for the International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risks to Humans. This important and prestigious series reviews and evaluates specific exposures and chemicals for evidence of carcinogenicity. Each committee member reviews a significant aspect of the literature and prepares a lengthy and detailed written

document. These documents are reviewed and discussed in a 10 day meeting in Lyon, France resulting in an evaluation of carcinogenic activity to humans and ultimately the publication of a monograph. The series is currently in Volume 127. I served on the Monograph Committees on "Tobacco Habits Other than Smoking," Volume 37, 1985; "Tobacco Smoke and Involuntary Smoking", Volume 83, 2002; "Betel Quid and Areca Nut," Volume 85, 2003, as committee Chair; "Smokeless Tobacco and Some Related Nitrosamines," Volume 89, 2004, and "A Review of Human Carcinogens; Lifestyle Factors," Volume 100E, 2009. I was a member of the National Toxicology Program Board of Scientific Counselors from 1997-2001 and the Science Advisory Board for the National Center for Toxicological Research from 1998-2002. I was on the Board of Scientific Counselors for the Division of Cancer Etiology of the National Cancer Institute from 1989-1995. I was Chair of the Division of Chemical Toxicology of the American Chemical Society from 1999-2000. I served on the Health Research Committee of the Health Effects Institute from 1992-1996. I have also served on numerous advisory groups for academic research centers specializing in toxicology and cancer research.

I am in demand as a speaker on topics pertinent to cancer prevention research, with particular emphasis on tobacco and cancer including basic, applied, and epidemiologic studies on nitrosamines. I have given formal invited lectures worldwide averaging about five per year since 2002 in almost every state of the U.S. and at scientific conferences and universities in Europe, Asia, and South America.

I have published over 880 original manuscripts, book chapters, reviews, and other peer reviewed documents in the scientific literature. This includes more than 600 original research articles in peer-reviewed journals. More than half of these original research articles are concerned with nitrosamines, including nitrosodimethylamine (NDMA), also known as dimethylnitrosamine (DMN). My H-index is 91, and my articles have been cited more than 35,000 times.

My first publication on nitrosamines was in 1974 when my colleagues and I discovered *N'*-nitrosonornicotine (NNN) in smokeless tobacco. This was the first example of a carcinogenic nitrosamine in unburned tobacco; in fact, the first example of any carcinogen in unburned tobacco. This paper was published in *Science*, and revolutionized the characterization and carcinogenicity assessment of tobacco products.

In the 1970s when my research on nitrosamines began, there was great interest in these compounds as potential carcinogenic constituents of food, drugs, tobacco products, and other consumer products. In 1956, Magee and Barnes had published their groundbreaking study demonstrating that NDMA caused liver cancer in rats. This was remarkable because NDMA is a water-soluble compound with only 11 atoms, and it had been supposed at the time, based on studies of polycyclic aromatic hydrocarbons, that carcinogenic agents were typically larger fat soluble compounds. The Magee and Barnes study stimulated an explosion of research on nitrosamines including extensive carcinogenicity testing and analysis

projects. Beginning in the 1970s, the U.S. Food and Drug Administration held regular meetings to address critical issues such as the contamination of bacon with *N*-nitrosopyrrolidine and related volatile nitrosamines including NDMA. Numerous analytical methods were developed for volatile nitrosamines such as NDMA in particular. The challenge was to be able to quantify relatively low levels of nitrosamine contaminants in common food and drug products. This was before the development and common use of highly sensitive mass spectrometers which are the current instruments of choice for the routine analysis of nitrosamines. Various methods were developed, but the one that was ultimately widely used was "Thermal Energy Analysis," in which the nitrosamine molecule was split and the released NO detected. This method was developed by a small company - Thermo Electron Corporation - now the huge instrument and scientific products corporation Thermo Fisher Scientific. The Thermal Energy Analyzer method was applied to numerous products and NDMA contamination was found in products such as beer, cured meats, and others. There was sufficient interest in nitrosamines in the period 1973-1996 that the International Agency for Research on Cancer sponsored biennial meetings for presentation of research on nitrosamines. These meetings were typically attended by 200-300 participants. Ultimately, the levels of common volatile nitrosamines such as DMN in most consumer products were decreased by process modifications and their concentrations rarely exceeded 5-10 ppb (0.005-0.01 ppm).

Our research on carcinogens in tobacco products fit very well into this framework of international interest, and we pursued it aggressively. Using my skills in analytical organic chemistry and mass spectrometry, and working together with an outstanding interdisciplinary team at the American Health Foundation, we carried out studies on the analysis of nitrosamines in tobacco products, the formation and synthesis of tobacco-specific nitrosamines, and the carcinogenicity of nitrosamines including tobacco-specific nitrosamines, cyclic nitrosamines, and other nitrosamines found in consumer products. We then extended our studies to investigate the metabolism of nitrosamines in laboratory animals and humans, and based on these studies developed biomarkers of nitrosamine exposure by quantifying nitrosamine metabolites in human urine. This research led to further investigations of human exposure to nitrosamines, particularly tobacco-specific nitrosamines. In a series of studies with significant policy implications, we demonstrated consistent exposure of non-smokers to carcinogenic tobacco-specific nitrosamines such as NNK via secondhand tobacco smoke in the home and a variety of commercial settings. These studies analyzed urinary NNAL, a metabolite of the lung carcinogen NNK, and contributed significantly to research supporting the clean air acts that have virtually eliminated indoor smoking, a known cause of lung cancer in non-smokers. Our studies on nitrosamine metabolism led logically to research on the interaction of their metabolites with DNA, the critical step in cancer induction by nitrosamines and other carcinogens. Our group characterized most of the DNA adducts formed by tobacco-specific nitrosamines and related cyclic nitrosamines. This research took advantage of my strong background in organic chemistry and analytical chemistry, particularly with the application of mass spectrometry.

Thus, as a result of more than 45 years of research in chemical and tobacco carcinogenesis, much of it focused on nitrosamines, I am thoroughly familiar with the state of the art in the formation, quantitative analysis, chemistry, biochemistry, metabolism, carcinogenicity, human exposure biomarkers, and DNA damage by nitrosamines. I currently serve on the European Food Safety Authority panel evaluating nitrosamines in food. I also served on the expert panel for the FDA Workshop entitled "Nitrosamines as Impurities in Drugs: Health Risk Assessment and Mitigation Public Workshop," March 29, 2021.

II. Nitrosamines

1. Chemical structures.

Nitrosamines are simple organic compounds formed by the attachment of an N=O group to an amino nitrogen.

2. Formation of nitrosamines

The formation of nitrosamines from secondary amines is textbook organic chemistry, a reaction familiar to all students in their first encounter with organic chemical reactions.[1,2] The nitrosation of secondary amines occurs so easily that it was once widely used in qualitative organic analysis as a test for the presence of a secondary amine, but after the discovery of nitrosamine carcinogenesis, this was eventually discontinued. Secondary amines such as dimethylamine and diethylamine are easily nitrosated by the agent nitrous anhydride (N_2O_3), which is formed from 2 molecules of nitrous acid (HNO_2), the conjugate acid of sodium nitrite ($NaNO_2$).[3] N_2O_3 reacts rapidly with a secondary amine such as dimethylamine or diethylamine to form the corresponding nitrosamine, in this case NDMA or *N*-nitrosodiethylamine (NDEA), respectively. The optimal pH for this sequence of reactions is 3.4, but it occurs over a wide range of pH values including at a pH 7 (neutral) with varying rates as expressed by the equation:

Rate = k [amine][nitrite]^2.

Tertiary amines can also be nitrosated to form dialkylnitrosamines such as NDMA.[4] Nitrosation of nicotine to produce NNK and NNN is a well-known example of tertiary amine nitrosation.[5] Nitrosation reactions can occur at neutral and basic pH with catalysis by formaldehyde[6] and can be inhibited by ascorbic acid.[7] Regarding the formation of NNK and NNN, we applied the known nitrosation chemistry to demonstrate that nicotine could be converted to 3 nitrosamines - NNK, NNN, and NNA.

This groundbreaking research established the chemical basis of nicotine nitrosation that eventually led to the identification of NNK in tobacco and tobacco smoke.[8,9] The identification of NNK in tobacco products then led to its testing for

carcinogenicity, which showed that it is a potent lung carcinogen in multiple animal species, independent of the route of administration, inducing mainly adenocarcinoma of the lung, now the major type of tumor seen in cigarette smokers.[10]

3. Analysis of nitrosamines

A great deal of effort has been devoted to the analysis of nitrosamines in various settings including food, drinking water, tobacco products, beer, medicines, and other consumer products. The rationale for these detailed and extensive studies derives from the powerful carcinogenicity of these compounds, for which the scientific community has consistently raised concerns about human exposure. Highly reliable analytical methods for determination of trace amounts of nitrosamines have existed for decades – first the nitrosamine specific "Thermal Energy Analysis" noted above and in more recent years sophisticated and sensitive mass spectrometric methods. All of these methods have been extensively validated for accuracy, precision, sensitivity, and overall reliability, and all existed prior to and during the development of the manufacturing processes at issue. The earlier analyses of preformed nitrosamines in food and beverages have been reviewed. In a representative summary, levels of "volatile nitrosamines" such as NDMA and NDEA in at least 60 different food types were recapitulated, typically being found in the 0-10 parts per billion range (micrograms per kilogram, or micrograms per liter), with occasional exceptions often involving foods preserved by smoking or related techniques. Levels of volatile nitrosamines in food are now generally lower in part because of regulations regarding the amount of nitrite that can be used.[11] A recent review has summarized current analytical data on human exposure to preformed nitrosamines.[12,] Nitrosamine levels in tobacco, food and beverages, drinking water, and personal care products were presented. The highest average levels were found in tobacco products (16,100 ng/g), followed by personal care products (1500 ng/g), while the lowest amounts were found in food and beverages (6.7 ng/g). Maximum average exposure to nitrosamines was estimated at about 25 µg per day, driven mainly by use of tobacco products.

4. Carcinogenicity of nitrosamines and NDMA in particular

In a landmark publication, Magee and Barnes first demonstrated the carcinogenicity of NDMA.[13] The substance was administered in the diet of rats (10 male and 10 female) at a concentration of 50 ppm. Between the 26th and 40th week, 19 of the treated animals developed primary hepatic tumors, with metastases in 7 cases. This remarkable finding initiated an entire branch of research ultimately resulting in the discovery of nitrosamines that readily and specifically induced tumors in virtually all major organs. High doses of NDMA are lethal; a median lethal dose in rodents of 20-40 mg/kg body weight has been reported.[14] The principal mechanism of death is severe hemorrhagic necrosis of the liver.[15] Consistent with this, cases of human poisoning by NDMA have been reported when large amounts of

the compound were used without precautions or when NDMA was used in deliberate attempted murders.[16]

The carcinogenicity of NDMA was demonstrated in several different strains of rats. Long-term administration of non-lethal doses of NDMA, typically about 4 mg/kg bw/day, consistently produced high incidences of hepatocellular carcinomas and cholangiocellular tumors. Short term administration of high doses of NDMA typically produced kidney tumors in multiple studies. The carcinogenicity of NDMA was significantly reduced by substitution of its methyl hydrogens with deuterium; the resulting deuterium isotope effect retarded its metabolic activation.[17] An extensive dose-response study of NDMA and NDEA on 4,080 rats demonstrated that a dose of 1 ppm of NDMA or NDEA in the drinking water caused about 25% of the rats to develop a liver neoplasm, a dose of 0.1 ppm caused about 2.5% to do so, and a dose of 0.01 ppm caused about 0.25% to do so, etc., with no indication of a "threshold."[18] In a study carried out by our group, the carcinogenic activities of NDMA and the tobacco-specific lung carcinogen NNK were compared.[19] Groups of 30 male F-344 rats were given 60 s.c. injections of 0.0055 mmol/kg of either NNK or NDMA over a 20 week period (total dose, 0.33 mmol/kg) and the experiment was terminated after 104 weeks. NDMA induced liver tumors in 6 of 30 rats; NNK induced a similar number of liver tumors but also a high incidence of lung adenocarcinoma and nasal cavity tumors.

The carcinogenicity of NDMA has been demonstrated in multiple species.[20] In Syrian golden hamsters, it induced various types of liver tumors when given by gavage or in the drinking water. Chinese hamsters and European hamsters also developed liver tumors when administered NDMA by injection. Guinea pigs developed liver tumors when given NDMA in the diet. Rabbits given NDMA in the diet developed liver carcinomas with lung metastases. Rainbow trout given NDMA in the diet developed hepatocellular tumors. Various strains of mice injected with NDMA developed liver and lung tumors. Liver tumors were also observed in mastomys administered NDMA by subcutaneous injection. Guppies and frogs exposed to NDMA in aquarium water resulted in the development of liver tumors. Rabbit, mink, blue fox, and duck are additional species in which NDMA induced liver tumors.

The pharmacokinetics and DNA binding of NDMA have been studied in detail in a range of species including mice, rats, rabbits, hamsters, dogs, pigs, and monkeys. Consistently, these studies have demonstrated high systemic clearance and high oral bioavailability of NDMA. In one study, NDMA was rapidly excreted into the saliva after i.v. and p.o. administration to dogs.[21] A consistent and linear pharmacokinetic and metabolic pattern emerged in these studies resulting in the conclusion that extrapolation to humans of conclusions obtained in studies using laboratory animals was justified.[22,23,24,25]

Similarly, the carcinogenicity of NDEA, which is more potent than NDMA, has been demonstrated in multiple species including in various different strains of rat,

mouse, and hamster as well as guinea pigs, chickens, rabbits, cats, dogs, pigs, monkeys, gerbils, snakes, hedgehogs, grass frogs, birds, and fish.[26] Dr. Lance Molnar, Ph.D., Mylan's Senior Director, Global Pharmacology and Toxicology, testified in his deposition that both NDMA and NDEA "are genotoxic carcinogens … in every experimental animal that they've been evaluated in" and are "demonstrated to produce tumors."[27]

In summary, NDMA and NDEA have been extensively tested in multiple species and at extremely low doses. Very few, if any, other chemicals have been so thoroughly tested for carcinogenicity, producing uniformly positive results. These data leave no doubt as to the high potency of NDMA and NDEA to induce tumors in laboratory animals and likely in humans. The lethality of NDMA at high doses has been observed in both laboratory animals and humans.

It is worth noting that both NDMA and a structurally related compound, dimethyl sulfate, are classified by IARC as belonging to Group 2A, "probably carcinogenic to humans."[28,29] According to the IARC Monographs preamble, this category is used when there is limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals. Of importance, IARC classification does not consider or indicate the strength of carcinogenicity. Thus, while NDMA is a far more potent carcinogen than dimethyl sulfate, both are classified as Group 2A. In an interesting exchange between Min Li of ZHP and ZHP's consulting toxicologist, Charles Wang, Ph.D., Dr. Wang advised that NDMA should actually be classified as Class 1B, stating: "Looks like IARC does consider NDMA as a Class 2A agent. However, according to the definition of Class 2 in ICH M7(R1) guideline, the Class 2 compound should be a 'Known mutagens with unknown carcinogenic potential (bacterial mutagenicity positive*, no rodent carcinogenicity data)'. There are plenty rodent carcinogenicity data for NDMA (see revised report in the attached, page 4). In Fisher MSDS, NDMA has been classified as Class 1B for carcinogenicity (attached)."[30] In addition, NDMA is not classified in Group 1, "carcinogenic to humans", as there is no instance, other than the poisoning murder incidents noted above, in which humans have been exposed exclusively to relatively low doses of NDMA in the absence of other potentially carcinogenic exposures; exposure to nitrosamines always occurs in mixtures. The exception to this IARC classification is the tobacco-specific nitrosamines NNK and NNN, to which human exposure also occurs as mixtures with other tobacco constituents (their carcinogenic activities and concentrations in tobacco products, particularly smokeless tobacco, along with sufficient metabolic data, lead to the higher classification).[31] Carcinogenicity data for dimethyl sulfate were summarized in the IARC Monographs.[32] Of course, as recognized below, it would be unethical to perform studies on the effects of NDMA and NDEA on humans due to their potency, consistent with the routine use of nitrosamines to cause cancer in laboratory animals.

More than 200 nitrosamines of different chemical structures (e.g., different R groups attached to the N-N=O group) have been tested for carcinogenicity and at

least 199 of them cause tumors in laboratory animals.[33] Nitrosamines are frequently organospecific in these studies, meaning that, depending on the structure of the nitrosamine, specific organs or tissues may be affected. Thus, for NDMA, the main target tissues demonstrated in animal studies are the liver and kidney, independent of the route of administration and species in which the test is performed. NDEA also targets these tissues in addition to causing tumors of the esophagus. In contrast, di-*n*-butylnitrosamine causes mainly tumors of the urinary bladder in several different animal species, while methylbenzyl nitrosamine specifically causes esophageal tumors in rats. These organospecific characteristics of nitrosamine carcinogenesis have been linked to their metabolism in specific tissues. Metabolism is absolutely required for the carcinogenicity of nitrosamines and it has been established that α-hydroxylation (replacement of the hydrogen atom adjacent to the N-N=O group by a hydroxyl group) catalyzed by specific cytochrome P450 enzymes present in the liver and other tissues, is the major pathway of metabolism leading to carcinogenesis. Multiple human tissues contain these enzymes and can metabolize nitrosamines; therefore, it is likely that when exposed to nitrosamines, humans are susceptible to developing a wider spectrum of cancers targeting additional organs. There are some instances of concordance between target tissues of nitrosamine carcinogenesis in laboratory animals and in humans. One example is the tobacco-specific nitrosamine NNN, mentioned above. NNN causes esophageal and oral tumors in rats. A prospective epidemiology study carried out in male cigarette smokers in Shanghai demonstrated a strong relationship between NNN exposure (as determined by NNN in urine) and esophageal cancer in the study subjects. NNN is also the only strong oral carcinogen in smokeless tobacco, a known cause of oral cancer in humans.

5. Carcinogenicity of nitrosamines in humans

Exposure to nitrosamines, including NDMA and NDEA, is a likely cause of cancer in humans. For example, there are examples of human poisoning by NDMA which coincide with toxicity studies in rats, as noted above. Human metabolism of NDMA, NDEA, and other nitrosamines by the pathways known to lead to DNA damage - identical to that seen in rats that developed tumors upon treatment with these nitrosamines - has been demonstrated in numerous studies using various experimental systems. These included human liver slices, human liver subcellular fractions such as microsomes (with activity just as high as rat liver microsomes), and explant cultures of various human tissues including bronchus, esophagus, bladder, and colon.[34] These results are consistent with the known activities of human hepatic cytochrome P450 enzymes such as P450s 2E1 and 2A6, which efficiently metabolize NDMA and NDEA.[35] DNA adducts known to result from NDMA and NDEA such as 7-methylguanine and O^6-methylguanine have been detected in human tissues.[36,37,38]

Thus, pathways of metabolism and DNA damage observed in humans clearly replicate those in laboratory animals that developed tumors upon treatment with NDMA. There is no reason to doubt that humans are susceptible to carcinogenesis

by NDMA and NDEA, considering their powerful carcinogenicity and the immense amount of supporting biochemical and toxicological data which are available. For example, among all nitrosamines, the tobacco-specific nitrosamines NNN and NNK are widely recognized as human carcinogens because of the high levels of human exposure.[39,40,41] In a study of exposures to British workers in the rubber industry, it was concluded that, "Consistent with previous studies, *N*-nitrosamines exposures in the rubber industry, were associated with mortality from cancers of the bladder, lung, stomach, leukaemia, multiple myeloma, oesophasus, prostate, pancreas and liver."[42]

Collectively, these observations support the human carcinogenicity of nitrosamines in general, which are potent mutagenic carcinogens. There is no reason to expect that humans would differ from laboratory animals with respect to the existence of nitrosamine carcinogenesis. All of the main metabolic activation pathways of nitrosamines that occur in laboratory animals treated with these compounds also occur in human tissues. The DNA adducts that are formed are identical. For example, treatment of laboratory animals with NDMA causes the formation of 7-methylguanine and O^6-methylguanine in DNA; the latter is known to cause mutations, specifically G to A mutations.[43] The exact same DNA adducts and mutations are found in human tissues exposed to NDMA in vitro. Given sufficient exposure to NDMA and NDEA, as with the levels found in the contaminated valsartan (see below), the formation of these DNA adducts would be sufficient to cause mutations and cancer in exposed humans.

The World Health Organization published a peer reviewed analysis of the carcinogenicity of NDMA in 2002.[44] The findings include: "Based upon laboratory studies in which tumours have been induced in all species examined at relatively low doses, NDMA is clearly carcinogenic and clastogenic. …. Qualitatively, the metabolism of NDMA appears to be similar in humans and animals; as a result, it is considered highly likely that NDMA is carcinogenic to humans, potentially at relatively low levels of exposure."[45] In the Effects Evaluation, with regard to Carcinogenicity, the study states: "The weight of evidence of the carcinogenicity of NDMA in mammalian species is consistent and convincing. Moreover, the pattern of tumour development is characteristic of that for a mode of action of carcinogenesis involving direct interaction with genetic material. In available studies, NDMA has induced tumours in all species examined (mice, rats, hamsters), at relatively low doses in some cases, irrespective of the route of exposure (oral, inhalation); tumours were induced in a wide range of tissues, including the liver, Leydig cells, lungs, kidney, and nasal cavity, in the absence of significant non-neoplastic effects, in the limited number of studies in which these were well examined. …. NDMA has been consistently mutagenic and clastogenic in human and rodent cells exposed *in vitro*. …. DNA adducts (in particular, O6-methylguanine) formed by the methyldiazonium ion generated during metabolism likely play a critical role in NDMA carcinogenicity. …. Putative pathways for the metabolism of NDMA are similar in rodents and humans, and indeed the formation of O6-methylguanine has been detected in human tissues exposed to NDMA. Therefore, owing to the

considerable evidence of carcinogenicity of NDMA in laboratory species, evidence of direct interaction with DNA consistent with tumour formation, and the apparent lack of qualitative species-specific differences in the metabolism of this substance, NDMA is highly likely to be carcinogenic to humans."[46]

ZHP cited to the WHO article in its Deviation Investigation Reports, and Min Li, Ph.D., Vice-President for Analytical Operations for ZHP, confirmed that this was because the article was considered to be scientifically reliable.[47] Dr. Li, who holds a Ph.D. in Organic Chemistry from Johns Hopkins University, also confirmed that ZHP stated in its own Deviation Investigation Report that NDMA is, "a probable, you know, carcinogenic to human."[48] ZHP also stated in the Deviation Investigation Report for the TEA process that "NDEA is considered as a probably human carcinogen based on projection from the animal studies." ZHP cited to Pharmol. Ther., 1996, Vol. 71, Nos. 1/2, pp. 57-81 for this. ZHP also cited to Int. J. Biol. Sci. 2013, Vol. 9, No. 3, pp.237-245 for the observation that NDEA "is one of the most potent chemical hepatocarcinogens of this class, which can induce a variety of liver lesions in rodents."[49] Min Li also confirmed that there are no studies deliberately performed on humans with regard to the carcinogenicity of nitrosamines because it would be unethical to knowingly give NDMA to humans, as a result of the risk of cancer. More to the point, Min Li confirmed that it would be unethical "to give humans NDMA in the levels that were found in the valsartan pills."[50]

Min Li also testified with regard to information provided to ZHP by ZHP's consulting toxicologist, Charles Wang, Ph.D. Dr. Wang advised ZHP regarding the risk associated with the NDMA and NDEA in the valsartan, and his analysis was the basis for the toxicological assessment found in the Deviation Investigation Reports.[51] Min Li confirmed that Dr. Wang was consulted because he was deemed an expert who would be trusted to provide "reliable information."[52] Among other things, Dr. Wang advised ZHP that the classification of NDMA as a Class 2A agent was incorrect, and should instead be designated as Class 1B, since, "There are plenty rodent carcinogenicity data for NDMA."[53] In addition, Dr. Wang consulted what he termed, "a carcinogenicity expert consultant to perform the analysis who knows risk assessment of carcinogen and kept updated in regulatory guideline and standards in this field." In an email dated July 6, 2018, this expert, James McDonald, Ph.D., advised Dr. Wang – who relayed this information to Min Li - that, "the body of evidence on this suggests pretty clearly that this is a likely human carcinogen at sufficient exposures. The argument that the company would have to make to keep this product on the market will be very difficult with this profile. I'm not exactly sure where one would begin given the very high levels [his understanding was 30 ppm per a prior email from Dr. Wang] you think they are seeing. …. I expect this is not what they would want to hear but, unless there is a compelling reason to leave this product on the market (e.g.: only product available to treat a serious, life-threatening disease), I would expect the FDA would ask for a recall."[54]

Bandaru Venkata Ramarao, Vice President of Quality Control for Hetero Unit 5 (the finished dose manufacturing division of Hetero) also testified to the

significant carcinogenic risk presented by the NDMA contamination of Hetero's valsartan. In discussing the FMEA (Failure Modes and Effects Analysis) risk evaluation performed by Hetero, Mr. Ramarao testified that the severity of the hazard presented by the NDMA impurity, "was at the highest level because of the level of NDMA and because it's a probable carcinogen. . ." The Health Hazard was described in the FMEA as "Identified impurity is carcinogenic in nature," and he agreed this meant, "this is something that can cause cancer. . ." Finally, he confirmed that the overall risk priority number, or RPN, was the maximum possible score of 125, meaning it was deemed, "intolerable."[55] Mr. Ramarao was also asked about the 2002 WHO publication discussed above, and agreed that, "Because of these health effects that we are talking about and the risk of cancer, it would never be acceptable to knowingly sell valsartan containing NDMA... ....[T]hat's the reason why the valsartan that was sold by Unit 5 with the levels of NDMA that were seen, that never would have knowingly been done if you had known the NDMA was there because of that health risk."[56] Mr. Ramarao agreed that due to the GMP failures that resulted in the NDMA contamination, "the result of that was that NDMA ended up in the valsartan, which is something that causes cancer," and the risk, "was assessed at the highest level because people ingesting NDMA at the levels that were found in these pills is something that will increase their risk for cancer...."[57]

The likely carcinogenicity of NDMA (and NDEA) in humans is also demonstrated by regulatory guidelines. For example, the 2013 ICH M7 Draft Consensus Guideline, and 2015 ICH M7 Guidance for Industry, titled Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, include *N*-nitroso compounds in the "cohort of concern" of "high potency mutagenic carcinogens" that are excepted from the acceptable intake levels set forth for DNA reactive substances.[58] The same is found in the December 2008 FDA Draft Guidance for Industry titled Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches: "However, there are some compounds containing certain structural groups (aflatoxin-like-, *N*-nitroso-, and azoxy-structures) that have extremely high carcinogenic potency and are excluded from the threshold approach."[59] The EMA takes the same approach. In discussing a group of "high potency genotoxic carcinogens" including nitrosamines, the "Guidelines on the Limits of Genotoxic Impurities" in effect from January 2007 to January 2018, states in part: "Some structural groups were identified to be of such high potency that intakes even below the TTC would be associated with a high probability of a significant carcinogenic risk."[60]

Lance Molnar, Ph.D., Mylan's Senior Director, Global Pharmacology and Toxicology,, agreed in his deposition that nitrosamines are treated as non-threshold by "the EMA, FDA, ICH … regulatory bodies in general" and that "non-threshold effect would mean that a single molecule could be detrimental."[61] Accordingly, Mylan's Toxicology report stated that "[t]his potential for carcinogenic activity is considered the critical effect of these compounds (*N*-nitrosamines) as it can theoretically occur at doses far lower than those required to produce alternative

toxicities after either acute or repeated exposures."[62] Similarly, Mylan's Medical Risk Assessment stated the following: "the potential risk associated with potential exposure to NDEA above the defined specification is significant and [risk of harm to patients] cannot be excluded."[63]

Studies published in the dietary literature are of course quite significant to this analysis. One study concluded, "our results suggested that there was a positive association between NDMA intake and gastrointestinal cancer risk, specifically of rectal cancer."[64] Another study recognized "challenges to nutritional epidemiological research on the relationship between dietary nitrosamines and cancer occurrence," but found "an increased risk of colorectal cancer among individuals with a high intake of NDMA."[65] The authors of yet another study concluded in part, "According to our study, processed meat intake was positively associated with cancers of the oesophagus, stomach, colon, rectum, larynx, lung, breast, prostate, and urinary bladder. Therefore, processed meat could be said to act as a multi-organ carcinogen among humans." The authors pointed to nitrosamines as a potential causative agent.[66]

This body of literature also includes studies cited and analyzed in the 2002 WHO article discussed above, which includes Risch, H.A., Jain, M., Choi, N.W., Fodor, J.G., Pfeiffer, C.J., Howe, G.R., Harrison, L.W., Craib, K.J., and Miller, A.B. (1985) Dietary factors and the incidence of cancer of the stomach, *Am J Epidemiol. 122*, 947-59; González, C.A., Riboli, E., Badosa J., Batiste, E., Cardona, T., Pita, S., Sanz, J.M., Torrent, M., and Agudo A. (1994) *Nutritional factors and gastric cancer in Spain, Am J Epidemiol. 139*, 466-73; La Vecchia, C., D'Avanzo, B., Airoldi, L., Braga, C., and Decarli, A. (1995) Nitrosamine intake and gastric cancer risk, *Eur. J. Cancer Prev. 4*, 469-74; Pobel, D., Riboli, E., Cornée J., Hémon, B., and Guyader, M. (1995) Nitrosamine, nitrate and nitrite in relation to gastric cancer: a case-control study in Marseille, France, *Eur J Epidemiol. 11*, 67-73; Rogers, M.A., Vaughan, T.L., Davis, S., Thomas, D.B. (1995) Consumption of nitrate, nitrite, and nitrosodimethylamine and the risk of upper aerodigestive tract cancer, *Cancer Epidemiol Biomarkers Prev. 4*, 29-36; Goodman, M.T., Hankin, J.H., Wilkens, L.R., and Kolonel, L.N. (1992) High-fat foods and the risk of lung cancer, *Epidemiology 3*, 288-99; De Stefani, E., Deneo-Pellegrini, H., Carzoglio, J.C., Ronco, A., Mendilaharsu, M. (1996) Dietary nitrosodimethylamine and the risk of lung cancer: a case-control study from Uruguay, *Cancer Epidemiol Biomarkers Prev. 5*, 679-82. In La Vecchia, et al., 1995, at 471, the authors concluded, "This study found a moderate but significant association between exogenous NDMA intake and gastric cancer risk. The association was consistently observed across strata of sex and age." In Gonzalez, et al., 1994, at 469, the authors commented on the association with gastric cancer, stating that they, "observed an increased risk associated with an elevated consumption of exogenous nitrosamines in both intestinal and diffuse types." In De Stefani, et al., 1996, at 681, the authors concluded, "In summary, NDMA intake was associated in this particular population with an increased risk of lung cancer." In Pobel, et al., 1995, at 70-71, the authors found: "In the present study we assess the risk of gastric cancer in relation to estimated dietary intake of nitrate, nitrite, and NDMA. The most important feature

revealed by this investigation is the increased risk associated with increasing intake of exogenous NDMA." In recognizing the complexity of the analysis, the authors concluded, "However, the findings presented here are consistent with the biological hypothesis and provide support for an association between nitrosamines and gastric cancer." In Goodman, et al., 1992, at 296, the authors concluded in part, "*N*-Nitroso compounds have been shown to be mutagens and important carcinogens for a number of target organs, such as the liver, stomach, brain, and lung. In this analysis, we found a strong relation between consumption of nitrite in men, and dimethylnitrosamines in men and women, and the risk of lung cancer." In Rogers, et al., 1995, at 33, the authors concluded in part that, "consumption of foods high in NDMA resulted in an elevated risk of cancer," in the "upper aero-digestive tract." In Risch et al., 1985, at 956, the authors concluded: "In summary, our data strongly suggest, in consonance with several previous studies, that nitrite intake is associated with risk of stomach cancer occurrence. Whether this relationship is mediated through the conversion of nitrite to *N*-nitroso compounds is unclear, although some protection appears to be afforded by consumption of citrus fruit."

To the extent these or other studies do not find a significant association, or raise questions, this can be explained by small or relatively small sample size, inadequate follow up period to capture all cancers, bias/inadequate dose quantification, potentially mitigating dietary factors such as vitamin C intake, and others.

Anticipating potential responses regarding proof of a cause and effect relationship, aside from the fact that a study cannot be ethically constructed to deliberately administer NDMA or NDEA to humans, this has been addressed. For example, in one article published in 1984 the authors noted the impediments to definitively proving cause and effect, "due to the insensitivity of the epidemiological instruments available today and to the lack of truly unexposed populations that could be used as controls." The authors stated in part, "Although a causal association between nitrosamine exposure and human cancer has not yet been rigorously established, the recognized association between exposure to nitrosamines in unburned tobacco products such as smokeless tobacco and oral cancer in humans is as close as one is likely to get in epidemiological studies of this class of carcinogens. In addition, biochemical, pathological, and experimental data provide little evidence that humans are resistant to the carcinogenic action of NOC [*N*-Nitroso compounds], from either preformed or endogenous sources… Although quantitative differences exist between rodents and humans in repair of DNA alkylation damage, the mechanisms of repair of this damage appear to be the same. Recently, malignant transformation of human pancreatic epithelial cells by NDMA has been reported."[67] A meta-analysis that noted varied results in studies that were reviewed, and the strengths and limitations of the study at hand, primarily based on difficulties in studying the effects of food intake, concluded in part that the risk of gastric cancer could be increased by "high consumption" of NDMA. The authors specifically noted: "When daily NDMA intake reached 0.12 ug, the harmful effect to human became more obvious."[68] For perspective, 0.12 ug (micrograms) is

equivalent to 120 ng (nanograms), and in a 320 mg dose of valsartan would equate to 0.375 ppm. These levels are in line with the levels established by the FDA, and were exceeded by the vast majority of the valsartan tested for nitrosamine contamination.

The EMA evaluated the valsartan contamination and concluded that the levels of NDMA and NDEA should be reduced to the maximum extent possible, but set thresholds consistent with the FDA's thresholds, factoring in the potential for nitrosamines to exist due to background levels for example in water. The statement firmly recognizes that there is no technically safe level, and the establishment of the thresholds is the product of a risk benefit analysis. Even though the EMA concluded that the risk is relatively low, that is also a finding of a real risk that is unacceptably dangerous, hence the establishment of the threshold levels.[69] Of interest as well, the EMA statement cites to and discusses a study performed in Denmark regarding the risk posed by the valsartan contamination, and found an increased risk for colorectal cancer and uterine cancer.[70] The EMA commented that the 4.6 year follow up interval was likely too short, and that the number of people studied too small, to draw any firm conclusions based on the data.[71]

A recently published study utilized data from a German health insurance database.[72] The cohort included those who filled at least one prescription of potentially contaminated valsartan from 2012 to 2017. The article refers to the establishment of whether or not the valsartan was manufactured utilizing API from ZHP, but without analysis of which manufacturing process was used. Thus, there is a concerning lack of data as to the extent to which those consuming potentially contaminated valsartan actually consumed contaminated valsartan. The endpoint was a cancer diagnosis. A statistically significant association for liver cancer was identified, but no association was identified for the overall risk of cancer or for other specific cancers. The authors pointed out that "molecular mechanisms known for NDMA in the pathogenesis of liver cancer in experimental animals support an association with NDMA exposure in humans. It may be that NDMA exposure promotes cancer development in already existing, as yet undiagnosed early stages and thus hastens clinical manifestation." They also recognized that, "The immediate recall of all potentially NDMA-contaminated valsartan drug products by regulatory authorities world wide was necessary in order to protect the public health." Thus, even with the limitations set forth in the study and discussed below, the study supports the conclusion that the contaminated valsartan increases the risk of cancer.

The study actually contrasts itself with Pottegard, discussed above in the context of the EMA statement, and points out the small number of people (5,150) evaluated in that study, and the small number of cancers in that study, and thus an inadequate sample size, by contrast to the large number of people studied in this study (780,871), which is a strength of the study. However, the study does recognize some significant limitations, including lack of information regarding the

NDMA content of the valsartan taken by those studied, and the short three year follow up.

Min Li, Ph.D. of ZHP testified with regard to a recent study published by Yoon et al. from South Korea.[73] Dr. Li testified suggested that this study supported the notion that NDMA would not increase the risk of cancer in a human.[74] The study addresses multiple limitations on its face and also suffers from a significant flaw that led the authors to retract the article. Yoon relied on a 2016 study by Zeng which found that "NDMA excreted in urine after ranitidine intake was 95.6 ng/mL, which was a 430-fold increase than before ranitidine use."[75] Yoon relied on those figures, and further added that "[a]ctual systemic NDMA exposure is likely much higher than that eliminated in urine."

The Zeng study collected urine samples of ranitidine users and tested the urine for NDMA. However, Zeng utilized GC-IT-MS for its analysis of urine samples. That test utilizes heat, and has been shown to cause residual ranitidine in the urine to form NDMA. Zeng was retracted on May 4, 2021 at the request of the authors: "[r]ecent research (1) has identified the potential for an analytical artefact associated with the use of gas chromatography that could have contributed to the levels of N-nitrosodimethylamine (NDMA) measured in urine samples containing ranitidine in this study. Given this artefact, the authors have informed the journal that their NDMA measurements are not reliable."[76]

On September 13, 2019, the FDA recommended the use of liquid chromatography with high resolution mass spectrometer (LC-HRMS) to measure levels of NDMA in ranitidine drug products, because gas chromatography (GC) based methods had been observed to elevate NDMA levels in tested materials.[77] On October 2, 2019, the FDA released a statement indicating that testing by LC-HRMS has shown the presence of much lower levels of NDMA in ranitidine.[78,79] On November 1, 2019, the FDA issued "Statement on new testing results, including low levels of impurities in ranitidine drugs." The statement indicated, "we have found levels of NDMA in ranitidine that are similar to the levels you would expect to be exposed to if you ate common foods like grilled or smoked meats. We also conducted tests that simulate what happens to ranitidine after it has been exposed to acid in the stomach with a normal diet and results of these test indicate that NDMA is not formed through this process. Similarly, if ranitidine is exposed to a simulated small intestine environment, NDMA is not formed." The FDA also stated that "[a]lthough many of these levels of NDMA observed through FDA testing are much lower than the levels some third-party scientists first claimed, some levels still exceed what the FDA considers acceptable for these medicines [96 nanograms of NDMA]."[80] The FDA tested various doses of ranitidine from eleven companies, three of which did not exceed 96 nanograms of NDMA.[81] None exceeded 1 microgram.[82] Thus, the study population included an unknown number of people who took uncontaminated valsartan. The Yoon study does not provide a compelling case to conclude that NDMA does not increase a human's risk of cancer when ingested at the levels seen with the contaminated valsartan.

III. Formation of Nitrosamines in the Valsartan API

As noted above, the formation of nitrosamines from secondary amines in the presence of nitrite and acid is absolutely basic organic chemistry. Any chemist who has taken even a basic organic chemistry course should know this. The test for a secondary amine was reaction with nitrite: a yellow oil, the nitrosamine, would be observed. A primary amine would produce bubbling due to the release of nitrogen from an unstable primary nitrosamine which rearranges to an unstable diazonium compound; a tertiary amine would not react (later shown to be not completely correct). Decades of research and volumes of published material clearly demonstrate that nitrite can react *easily* with amines to produce carcinogenic nitrosamines. The International Agency for Research on Cancer held international symposia on this issue every 2 – 3 years from the 1970s through the 1990s. The proceedings of these symposia are all available on the IARC website and describe hundreds of experiments demonstrating the ease of formation of carcinogenic nitrosamines in various settings including in chemical and drug manufacturing. For example, "It has been known since 1865 that the reaction of dimethylamine hydrochloride with sodium nitrite at an acidic pH yields *N*-nitrosodimethylamine."[83] Furthermore, the formation of nitrosamines in food cured with nitrite such as hot dogs and lunch meats has been extensively studied and documented. The U.S. Food and Drug Administration held regular meetings and symposia on this topic resulting eventually in significant decreases in the amounts of nitrite added to foods as a preservative. Volumes of research on nitrosamine contamination of various foods and tobacco products have been published. As set forth above, the FDA, ICH, and the EMA have all recognized the potential for nitrosamine impurities to exist in pharmaceuticals and the attendant risks. A qualified organic chemist in industry would be aware of this literature.

1. Nitrosamines in the ZHP API

I discuss the ZHP contamination in detail here to illustrate the root cause and the easy avoidability of the contamination. Ultimately, the manufacturers ignored basic chemistry principles, whether the root cause was reactions in the manufacturing process and/or cross-contamination due to solvent recovery or inadequate cleaning of equipment. The introduction of NDMA and NDEA into ZHP's Valsartan API was easily foreseeable. The Change Request Form for the Process Change for Valsartan Process II prepared on May 19, 2011, with an effective date of June 15, 2011[84] described the chemical processes, which included the addition of the solvent DMF, which was well known to decompose/degrade forming dimethylamine. "DMF decomposes slightly at its boiling point to afford dimethylamine and carbon monoxide, this reaction occurring even at room temperature in the presence of some acidic or basic materials."[85] "DMF ... Decomposes slightly at its normal boiling point to give small amounts of dimethylamine."[86]

There are multiple references in the Change Request Form to the need for process and method validation, and a reference to the need for testing including, "The residue of ZnCl2, and residue of solvents used in the process need to be tested for quality review." (Section 3). Unfortunately, from an organic chemistry perspective, ZHP failed to adequately evaluate the chemical processes, and failed to account for the risk of nitrosamine contamination. As a result, their risk assessment was inaccurate, i.e. "After evaluation, this change has a lower risk in terms of quality and safety,"[87] and "Synthetic route, intermediates remain the same and no adverse change in qualitative and quantitative impurity profile."[88] From the perspective of organic chemistry, as discussed herein and as recognized in ZHP's own root cause investigation (See ZHP Deviation Investigation Reports, ZHP00007221, PRINSTON0073443, PRINSTON0075797, PRINSTON0076100), a scientifically reasonable assessment of the Process Change would have identified the risk of formation of nitrosamine impurities, would have presumably led to testing for nitrosamines, and would have confirmed the formation was occurring. In addition, from an organic chemistry perspective this Change met the definition of a "Critical Change – A change which has direct or potential impact on product identity, strength, quality, purity and regulation, or have impact on validated Procedure, method, qualification or equipment."[89] This was a critical change because the process change had the foreseeable capacity to create, and resulted in, dangerous NDMA contamination. This analysis applies as well to the change from the TIN process to the TEA process with sodium nitrite quenching, which resulted in the formation of NDEA and NDMA. In light of the known potential results of the chemical processes, identification of the clearly foreseeable NDMA and NDEA impurity contamination could have been easily accomplished.

ZHP was certainly aware of the presence and significance of impurities in Valsartan API from the early days of their development of the original manufacturing process for Valsartan. For example, ZHP's knowledge of the significance of potential impurities was documented in the peer reviewed medical literature in 2006.[90] The article begins with a statement of the fundamental principle at the core of this litigation, that "The quality and safety of pharmaceuticals can be significantly affected by the presence of impurities. Consequently, the testing and establishment of limits for impurities in active pharmaceutical ingredients have become important initiatives by government and the pharmaceutical industry." The article, which included a co-author identified as an employee of ZHP, discussed available technologies for the detection and identification of impurities in API, in this instance Valsartan API.

Once the presence of NDMA was discovered, it was not difficult to determine the root cause. A July 27, 2017 email within ZHP refers to the root cause, specifically the fact that NDMA was known to occur in valsartan as a result of the use of sodium nitrite in the sodium azide quenching process, and that there was a need for, "the optimization of the valsartan sodium azide quenching process."[91] Dr. Li also confirmed that this was known to be a "common problem in the production and synthesis of Sartan APIs."[92] ZHP similarly concluded in a June 2018 document

summarizing the purported first detection of, and establishment of the root cause of the NDMA contamination, "this impurity is most likely generated during the 'azide quenching' by nitrous acid of the API manufacturing process."[93] The use of nitrite to decompose the azide reactant in the Valsartan synthesis process was a significant error due to the risk of nitrosamine formation, which should have been recognized. The use of nitrite should have raised a gigantic ***RED FLAG*** that nitrosamines could be present in the API. The same applies to the TEA process with sodium nitrite quenching.

Analysis of the Valsartan batches manufactured by ZHP with the zinc chloride process showed the presence of an unknown peak eluting just after toluene in the GC-MS analysis. On June 6, 2018, ZHP customer Novartis, which had contracted for further analysis of this API, notified ZHP that the unknown peak had been identified as NDMA. ZHP noted its failure to account for nitrosamines: "By looking into our CEP documents, it shows that NDMA is **not part of the controls in the current approved specifications** of the drug substance," and "Due to the fact that NDMA is a recently found **unexpected impurity** with the nature of probable carcinogen, and in order to understand the root cause for the occurrence of this impurity, ZHP has initiated root cause investigation."[94] It is important to note that ZHP's repeated statements that the NDMA was not known until June 2018 are contradicted by the July 27, 2017 email discussed herein, which not only references the fact that there was NDMA in the valsartan, but also the root cause tied to the sodium nitrite quenching.

The documents from ZHP clearly demonstrate how the formation of NDMA could have been avoided. They identified three critical factors: 1) use of dimethyl formamide in the tetrazole formation step, and the dimethyl formamide may have contained trace amounts of dimethylamine or the dimethylamine was formed during the process; 2) quenching of azide using nitrous acid (formed from nitrite under acidic conditions); and 3) quenching takes place in the presence of the product. ZHP concluded that NDMA was formed only when all 3 factors were present, based on extensive analysis by ZHP. Factor 2 should have raised a ***RED FLAG*** for the potential formation of nitrosamines. The contamination of dimethyl formamide with dimethylamine or the formation of dimethylamine during the process was foreseeable, and should have been evaluated. Factor 3 was shown to be critical to the problem; when the extraction of the product was performed prior to the addition of nitrite to quench the azide, no NDMA was observed, whereas in their original process, all samples were contaminated with NDMA. The results of the three factor analysis are perfectly clear and demonstrate a massive disregard for potential nitrosamine formation. Extraction prior to quenching would have been a simple remedy for the problem and should have been pursued. In their analyses of the product, they would not have identified NDMA in the chromatograms unless they were specifically looking for it, because the peaks would be too small. *But that is not a legitimate scientific excuse: ZHP should have been actively looking for nitrosamines based on the discussion above.* They could have used nitrosamine-selective methods such as combined gas chromatography-mass spectrometry for

this analysis. Using this or related methods, the detection of NDMA would have been straightforward.[95]

Prior to the process change from the "Process II" process to the Zinc Chloride process, which replaced Triethylamine with Zinc Chloride for the Tetrazole Ring Formation, and also replaced the original reaction solvent Toluene, with DMF (Dimethylformamide) and MTBE (Methyl tert-butyl ether), NDEA was similarly formed when 3 factors were present: 1) trimethylamine used as a catalyst for tetrazole formation; 2) nitrite used for decomposition of excess sodium azide; and 3) both factors 1 and 2 are together with the crude product. Lower amounts of NDEA were also formed due to trace amounts of diethylamine present in the trimethylamine used as a catalyst in the tetrazole formation step, and/or by direct nitrosation of trimethylamine. All of this was foreseeable, and if considered and tested for, the NDEA contamination would have been detected.

In the FDA inspection of ZHP,[96] numerous deficiencies were found, including 1)inadequate change control system; 2)inadequate validation program; 3)insufficient investigation of critical deviations; 4) the quality unit does not always fulfill the responsibilities of the quality unit; 5)cleaning procedures do not have sufficient detail; 6) equipment is not always of appropriate design;7)preventive maintenance procedures are not always adequate; 8) lubricants, heating fluids and coolants are not always food grade lubricants and oils; 9) sampling plans are not always scientifically sound; 10) stability studies are not always adequate; and 11) production deviations are not always thoroughly investigated. These deficiencies indicate a general disregard for potential problems in the manufacturing process, including the formation of nitrosamines. The report notes that while there is a procedure for investigating "out of specification/out of trend" deviations in the analysis of the product, it apparently is inconsistently applied.

In the specific investigation here, a peak eluting after the solvent toluene was ultimately definitively identified by an outside laboratory, using combined gas chromatography-mass spectrometry (GC-MS), as NDMA. The initial investigations disclosed by ZHP did not detect this peak due to errors in the headspace analysis process by its contractor, Zhejiang Haotian Testing and Technology Service, in which the vial was improperly crimped leading to non-detection of NDMA and any other peak.[97] This is apart from ZHP's confirmed knowledge at least as of July 2017 that there was NDMA in the valsartan.[98] The 5290 batches manufactured between 2016-2018 were reported to have contained an average of 57-64 parts per million of NDMA.

ZHP NDMA AND NDEA LEVELS:

The NDMA contamination levels confirmed in ZHP's contaminated valsartan were reported to range from 3.4 to 120 ppm, with variation between the East Zone and West Zone of the manufacturing facility, likely based on variations in the

production processes.[99] Additional documents establish even higher contamination levels.

In a document titled: Response to DMF Information Request Letter, ZHP provided the FDA with NDMA test results on residual solvents from three validation batches, as well as the NDMA Test Results for Batches Manufactured Using the ZnCl2 Process, presented as a chart of the results of testing of 783 batches manufactured between 12-28-2011 and 5-23-2018, with NDMA levels as high as 188.1 ppm.[100] These levels could have been established, for example using GC-MS, throughout the time that ZHP manufactured the valsartan API. This is demonstrated by the ease with which Novartis was able to identify NDMA. The same applies to the triethylamine manufacturing process with sodium nitrite quenching. In the Response to DMF Information Request Letter, ZHP reported NDMA levels for 55 batches that had been tested as of September 1, 2018, as high as 73.9 ppm.[101]

A separate spreadsheet provided by Solco to the FDA also documented the test results for the ZHP valsartan API batches manufactured with the zinc chloride process which were used to manufacture ZHP's finished dose for sale to Solco to be distributed in the United States, as well as the levels established by ZHP for the finished dose. There are a small number of batches with results in the single digits, with the lowest at 3.4 ppm, and the majority of the remaining batches have levels up to 188.1 ppm.[102] For context, 3.4 ppm translates to 1088 ng in a 320 mg pill, and 188.1 ppm translates to 60,192 ng in a 320 mg pill.

The ZHP Deviation Investigation Report dated November 11, 2018, titled Investigation regarding unknown impurity (genotoxic impurity) of Valsartan API (TEA process), provides NDEA levels for the TEA process valsartan API.[103] Testing of six validation batches established NDEA results of 0.03, 5.33, 12.77, 13.60, 18.83, and 13.51 ppm.[104] A separate table in that Report provides ranges and averages for the testing of 85 batches manufactured with the TEA process, documenting a range of 0.03-42.14, and average of 13.46, presumably in ppm. That table also sets forth NDEA levels in 111 batches manufactured with the zinc chloride process, documenting a range of 0-4.23 and average of 0.18, presumably in ppm.[105] As stated, since these impurities resulted from the manufacturing processes, all batches should be assumed to have been similarly contaminated, including those not tested.

Since we know that all batches of valsartan API manufactured with the zinc chloride process were contaminated with NDMA, the NDEA contamination would be additive and therefore further increase the risk of cancer for each pill manufactured from those batches. The TEA Deviation Investigation Report indicates that the likely cause of the NDEA contamination in the valsartan API manufactured with the zinc chloride process was cross-contamination due to shared equipment and solvent recovery.[106]

The aforesaid contamination of ZHP's valsartan API with nitrosamines including NDMA and NDEA, which are potent mutagenic carcinogens, resulted in an

unacceptable increased risk of cancer for those taking the medication. Thereafter, when aberrant peaks demonstrated unaccounted for impurities, the nitrosamine contamination could have been easily discovered based on knowledge of the potential chemical reactions and application of GC-MS to identify potential NDMA/NDEA. This was identified by Novartis even without the full information available to ZHP.[107] These failures and the consequent contamination of the Valsartan API resulted in the dangerous and unreasonable risk of causing or increasing the risk of causing cancer for those who ingested the contaminated valsartan with the reported levels of NDMA and NDEA.

No level of NDMA or NDEA in a pharmaceutical drug is "safe," in the sense that every exposure increases the risk to some incremental extent that one will develop cancer. The FDA set limits once the valsartan contamination was disclosed, and the aforesaid levels exceed the 96 nanogram/0.3 parts per million daily limit (based on 320 mg tablets) applied by the FDA to NDMA, and the 26.5 nanogram/.083 parts per million daily limit (based on 320 mg tablets) applied by the FDA to NDEA.[108] Those who ingested the contaminated valsartan above those levels sustained the unreasonably dangerous and unacceptable risk that this would cause or substantially contribute to causing cancer as a result of the NDMA and NDEA contamination.

It is important to note that the FDA's short term decision to delay the recall of the contaminated valsartan for a very brief period of time was not an endorsement of the safety of the medication.[109] Instead, this was the result of concern over the availability of the medication due to the widespread contamination, and a balancing of the risk of cancer against the more immediate risk of heart attack, stroke, or other life threatening results of a person abruptly ceasing the use of their hypertension medication.

2. Nitrosamines in the Hetero API

Hetero utilized a zinc chloride/DMF/sodium nitrite quenching process that was materially the same as ZHP's zinc chloride process, and the root cause of the NDMA impurity contamination of Hetero's valsartan was the same.[110] The reason for this occurring was the same as with ZHP. Mr. Ramarao confirmed this when he agreed with the following: "the most important problem" was that Hetero Unit 1 (API manufacturer) and Unit 5 (finished dose manufacturer) "never even realized the possibility that NDMA could form, so it was never actually looked for. That's the fundamental problem, correct?"[111]

The NDMA levels found on testing of Hetero's valsartan API manufactured with the zinc chloride process were confirmed in deposition testimony to range from 0.83 ppm to 7.78 ppm. This data was based on the testing of six batches, and was confirmed to be "representative of the contamination levels across the API – the Valsartan API that was sold from Unit 1 to Unit 5 and then sold in the United States."[112] As stated, since these impurities resulted from the manufacturing

process, all batches should be assumed to have been similarly contaminated, including those not tested.

These contamination levels caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan manufactured by ZHP.

3. Nitrosamines in the Aurobindo API

Aurobindo manufactured valsartan API for sale in the United States using a process referred to as the Toluene route, according to deposition testimony from Sanjay Singh, Associate President of North American Technical Operations.[113] The nitrosamine contamination of Aurobindo's valsartan API resulted from cross-contamination caused by a solvent vendor, Lantech. [114] The root causes of this cross-contamination included (1) a contaminated plate in a vertical heat exchanger that was shared between Aurobindo and Mylan, among others,[115] and caused residue to build up and carry over from batch to batch, causing NDEA contamination in the tri-*n*-butyl tin chloride[116] (Aurobindo's Chief Quality Officer analogized this to a dirty microwave),[117] (2) shared tanks (between Aurobindo, Mylan, and others) that were used to store the tri-*n*-butyl tin chloride resulting in NDEA contamination,[118] and (3) Lantech supplied the fresh solvent ethyl acetate which contained TEA,[119] and during the manufacturing process the TEA reacted with nitrosyl chloride, a byproduct of Aurobindo's API manufacturing process, resulting in NDEA contamination.[120]

Both NDMA and NDEA were detected in the valsartan API utilized by Aurobindo. The reported levels of NDEA ranged from 0.028 ppm to 1.508 ppm.[121] The levels of NDMA ranged from below .1 ppm to .129 ppm, and were additive to the NDEA levels, where present.[122] Assuming the same solvent related practices were utilized with both the tested and untested batches, all batches should be assumed to have been similarly contaminated, including those not tested.

These contamination levels caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan manufactured by ZHP.

4. Nitrosamines in the Mylan API

Mylan was vertically integrated and supplied valsartan API to Mylan's finished dose manufacturing facilities, and also supplied valsartan API to its sole external United States finished dose customer, Teva.[123] Mylan's root cause investigation found that NDEA was created in the solvent recovery process for o-xylene, the recovery layer of which contained traces of diethylamine and triethylamine, when it was recovered with nitrous acid, and carried over to the final API.[124] Mylan acknowledged that it was warned by its supplier as early as 2014 to

"avoid … nitrosating agents" with TEA due to the "possibility of formation of nitrosamines with nitrites or other nitrosating agents."[125]

Mylan confirmed that NDEA was present in every single API batch.[126] Mylan's API testing confirmed NDEA contamination in every API batch released to the US market, with levels between 0.1 ppm to 1.57 ppm.[127] Dr. Walt Owens, current Head of Global Regulatory Affairs and former Head of Global Quality, testified that "the API and finished dosage form [nitrosamine testing] results were essentially the same, you would be able to test the API alone."[128] Mylan's testing also showed that the valsartan API contained sporadic levels of NDMA contamination, in addition to the NDEA, including BQL, BDL, and from 0.01 ppm to 0.09 ppm.[129]Assuming the same solvent related practices were utilized with both the tested and untested batches, all batches should be assumed to have been similarly contaminated, including those not tested.

These contamination levels caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan manufactured by ZHP.

5. Nitrosamines in the Finished Dose Formulations

The NDMA and NDEA levels would be expected to be the same or nearly so in the finished dose formulations incorporating the contaminated valsartan API. This was addressed and confirmed in the deposition of Hai Wang, the President of Solco, ZHP's wholly owned distributor in the United States. Hai Wang confirmed that this was determined by ZHP and that data was provided to the FDA.[130] Both ZHP and Hetero were vertically integrated thus the above discussion of the causes and levels of the nitrosamine contamination of the API addresses the NDMA and NDEA contamination in their finished dose formulations as well.

Finished dose manufacturers Teva and Torrent obtained valsartan API from API manufacturers and then incorporated it into their finished dose formulations.

6. Nitrosamines in the Teva Finished Dose Formulation.

Teva manufactured and sold finished dose valsartan utilizing ZHP manufactured valsartan API, and Mylan manufactured valsartan API, labeled either as Teva or Actavis.[131] The valsartan finished dose labeled as Actavis and sold in the United States initially was manufactured using ZHP TEA process with sodium nitrite quenching valsartan API, and then ZHP zinc chloride process valsartan API beginning in late-2014.[132] The valsartan finished dose labeled as Teva was manufactured using Mylan valsartan API.[133]

ZHP reported NDMA levels to Teva between 0.8 ppm and 240.1 ppm.[134] Teva also tested 83 batches of ZHP valsartan API with NDMA levels of 30.01 ppm to 221.63 ppm.[135] In addition, Teva tested six batches of its finished dose valsartan

manufactured with ZHP valsartan API with NDMA levels of 14.8 ppm to 31.3 ppm.[136] It was confirmed that all ZHP valsartan API sold to Teva contained NDMA in excess of 0.3 ppm.[137] Daniel Barreto, Teva's former Senior Vice President Global Quality Compliance, testified that the finished dose product would have the same levels of NDMA as tested in the API and Teva "extrapolate[d] the nitrosamine test results of the API to the valsartan finished dose." [138]

Teva also tested for NDEA. Teva initially tested eleven batches of Mylan valsartan API and ten of the eleven batches had NDEA levels above 0.08 ppm, from 0.09 ppm to 0.50 ppm.[139] Teva tested 26 additional batches of Mylan valsartan API and twenty-four of the twenty-six batches tested above .08 ppm for NDEA, with results ranging from 0.08 ppm to 0.42 ppm.[140] Since the contamination occurred at the level of the API suppliers, the untested batches would be expected to have the same or very similar contamination levels as the tested batches, as discussed above.

These contamination levels caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan sold by Teva.

7. Nitrosamines in the Torrent Finished Dose Formulation.

Torrent purchased valsartan API from ZHP that was manufactured using the TEA with sodium nitrite quenching process. This was the only manufacturing process for ZHP valsartan API that was documented as being sold in the United States by Torrent.[141]

On August, 3, 2018, ZHP notified Torrent of "trace" amounts of NDMA in the Valsartan API sold to Torrent, which was the API manufactured using the ZHP TEA process with sodium nitrite quenching (as discussed above).[142] On Sept 7, 2018, ZHP notified Torrent that what ZHP described as, "another contaminant, NDEA, has been detected in the finished dose batches of valsartan."[143]

The levels of NDMA found on testing of the valsartan API purchased by Torrent from ZHP and then incorporated in its finished dose formulation sold in the United States were reported to range from 0.37 parts per million to 125.15 parts per million.[144] The levels of NDEA were found to range from 0.23 ppm to 16.93 ppm, with several batches found to be BDL and BQL range (Below Detection Limit and Below Quantification Limit).[145] All batches had NDMA and the majority had both NDMA and NDEA, which would increase the cancer risk. Since the contamination occurred at the level of the API supplier, ZHP, the untested batches would be expected to have the same or very similar contamination levels as the tested batches, as discussed above.

These contamination levels caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan sold by Torrent.

IV. Conclusion

The contamination of the valsartan API, and consequent contamination of the valsartan finished dose, as described above, caused an unreasonably dangerous and unacceptable risk of causing or substantially contributing to the causation of cancer for those ingesting the valsartan. As described above, a range of cancers have been associated with intake of NDMA (and NDEA by extension). In general, the increased risk would likely be commensurate with the contamination levels, dosages, and periods of use. Therefore, people who ingested the valsartan with higher contamination levels and larger doses, over longer periods of time, would likely have a more substantial increased risk as opposed to those who ingested valsartan with lower contamination levels and lower doses, and for shorter periods of use. However, even those lower levels, lower dosages, and shorter periods of use present an unreasonable danger and risk, a risk to which one would not knowingly or deliberately expose a person.[146]

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[54] *Ibid.*, 573:7-574:9, 622:3-648:5; CHARLESWANGO00289 (ZHP 316); CHARLESWANGO000447 (ZHP 319) (emails discussed in cited testimony).
[55] B.V. Ramarao Dep. Tr. 4/29/21, 259:20-268:4.
[56] B.V. Ramarao Dep. Tr. 4/30/21, 342:14-343:19.
[57] B.V. Ramarao Dep. Tr. 4/29/21, 377:5-20.
[58] (ZHP-310, p. 2).
[59] (ZHP-208, p. 8).
[60] (ZHP-206, p. 6).
[61] Lance Molnar Dep. Tr. 5/07/2021, 125-2-6; 121:22-23.
[62] (Pl-Molnar 5, at 2.).

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[74] Min Li Dep. Tr. 4/21/21, 334:18-339:4.
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[101] *Ibid.*, 9939-9940.
[102] Hai Wang Dep. Tr., 3/10/2021, 112:2-118:17; SOLCO00028261 (ZHP 118)).
[103] PRINSTON0075797.
[104] PRINSTON0075846.
[105] PRINSTON0075858.
[106] PRINSTON0075928-75937.
[107] ZHP00079913-45, at 9933-9938.
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https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan.

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[110] B.V. Ramarao Dep. Tr. 4/30/21, 372:15-373:23, 392:22-393:16.

[111] *Ibid.*, 425:16-24.

[112] *Ibid.*, 390:17-392:16.

[113] Sanjay Singh Dep. Tr. 5/20/2021, 32:24-33:9, 83:17-24.

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[115] *Ibid.*, 2021, 305:3-12.

[116] *Ibid.*, 180:4-181:7.

[117] *Ibid.*, 181:11-23.

[118] *Ibid.*, 204:10-24.

[119] *Ibid.*, 379:5-24.

[120] *Ibid.*, 380:1-18.

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[122] Auro-MDL-2875-0093561 (contains AuroLife batches); Auro-MDL-2875-0104586; Sanjay Singh Dep. Tr. 5/21/2021, 594:14-631:20.

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[124] Daniel Snider Dep Tr., 3/31/ 2021, 237:12-238:1, 260:16-262:14; Derek Glover, Dep Tr. 3/12/2021, 439:1-440:7.

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[126] Antony Gomas Dep Tr. 4/09/2021, 54:13-55:7; Daniel Snider Dep Tr. 3/31/2021, 196:6-22.

[127] (Pl-Gomas 5); Antony Gomas Dep Tr. 4/09/2021, 100:10-106:5 (confirming that Pl-Gomas 5 reflects the most comprehensive nitrosamine testing results for Mylan API and FD), 111:15-112:8 (plain valsartan), 112:19-113:15 (valsartan HCTZ), 114:4-12 (amlodipine valsartan); Daniel Snider Dep Tr. March 31, 2021, 196:6-22.

[128] Walt Owens Dep Tr. 4/21/2021, 79:15-81:2.

[129] (Pl-Gomas 5); Antony Gomas Dep Tr. 4/09/2021, 100:10-106:5 (see above); Snider Dep. 264:9-16 (stating that Mylan believed that dimethylamine present in the triethylamine yielded NDMA that carried over into the final API and FD).

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[131] Teva 230; Michelle Osmian Dep. Tr. 5/06/2021, 33:2-236:24; 239:7-240:2.

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[133] TEVA-MDL2875-00320639-673, at -639.

[134] TEVA-MDL2875-00546489.

[135] *Ibid.*

[136] *Ibid.*
[137] Claire Lyons Dep. Tr. 4/27/2021, 130:3-132:2.
[138] Daniel Barreto Dep. Tr. 4/14/2021, 201:23-202:9; 275:9-276:5; 367:9-368:2.
[139] TEVA-MDL2875-00048605, at 61 of 61.
[140] *Ibid.*, 58-59.
[141] TORRENT-MDL2875-00072650; Sushil Jaiswal Dep. Tr. 6/04/20211, 67:21-24, 68:1-7.
[142] TORRENT-MDL2875-00131255; Reddy Neravetla Dep. Tr. 5/26/2021, 102:2-21.
[143] TORRENT-MDL2875-00504834; Jocelyn Rivera Dep. Tr. 02/22/2021, 438:5-24; Dawn Chitty Dep. Tr. 5/13/2021, 349:13-24, 350:1-4.
[144] TORRENT-MDL2875-00366172; Sushil Jaiswal Dep. Tr. 6/04/2021, 64:5-22, 65:7-24, 71:7-23, 86:17-24, 87:1-19.
[145] TORRENT-MDL2875-00135398; Dawn Chitty Dep. Tr. 5/13/2021, 59:15-24, 61:14-18.
[146] As recognized above, the FDA allowed contaminated valsartan to remain available for a short time in order to ensure there would not a shortage of this blood pressure medication in the short term. This decision was not an indication that the ingestion of the contaminated valsartan was considered to be safe or desirable.

Exhibit 1

Stephen S. Hecht, Ph.D.

Winston R. and Maxine H. Wallin Land Grant Professor of Cancer Prevention
American Cancer Society Professor, American Chemical Society Fellow
Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

Education

Duke University, B.S. (with honors), Chemistry – 1964
Massachusetts Institute of Technology, Ph.D., Organic Chemistry – 1968

Professional Experience

Masonic Cancer Center, University of Minnesota, Minneapolis, MN

- Wallin Land Grant Professor of Cancer Prevention and Professor, Department of Laboratory Medicine and Pathology, 1996-present
- Head, Carcinogenesis and Chemoprevention Program, 1998-2014
- Member, Medicinal Chemistry and Pharmacology Graduate Programs, 1996-present

American Health Foundation, Valhalla, NY

- Director of Research, 1987-1996
- Chief, Division of Chemical Carcinogenesis, 1980-1996
- Head, Section of Organic Chemistry, Division of Environmental Carcinogenesis, 1973-1980

United States Department of Agriculture, Philadelphia, PA

- National Research Council Fellow, 1971-1973

Haverford College, Haverford, PA

- Assistant Professor of Chemistry, 1969-1971

Massachusetts Institute of Technology, Cambridge, MA

- Postdoctoral Fellow, Mass Spectrometry, Professor Klaus Biemann, 1968-1969

Honors and Awards

Academy for Excellence in Team Science, University of Minnesota, 2019
Listed in AACR Landmarks in Cancer Research, 2017: Tobacco-Specific Nitrosamines, *JNCI* 60: 819-824 (1978)
University of Minnesota Medical School Dean's Distinguished Research Lectureship, 2017
American Chemical Society Minnesota Section, Minnesota Award, 2017
University of Minnesota Medical School Wall of Scholarship, 2015
Elected American Association for the Advancement of Science Fellow, 2014
Selected as next Editor-In-Chief, *Chemical Research in Toxicology*, American Chemical Society, 2012
Joseph Cullen Award, American Society of Preventive Oncology, 2012
Elected American Chemical Society Fellow, 2009
Founders' Award, Division of Chemical Toxicology, American Chemical Society, 2009
Academy for Excellence in Health Research, Academic Health Center, University of Minnesota, 2006
American Association for Cancer Research-Cancer Research and Prevention Foundation Award for Excellence in Cancer Prevention Research, 2006
Merit Award, National Cancer Institute, 2004-2014
Dr. William Cahan Distinguished Professor Award, Flight Attendant Medical Research Institute, 2002
Alton Ochsner Award Relating Smoking and Health, 2001
American Cancer Society Research Professor, 2000-2009

Wallin Chair in Cancer Prevention, Masonic Cancer Center, University of Minnesota, 1996-
Endowed Chair in Carcinogenesis and Chemoprevention, American Health Foundation, 1992-1996
Cancer Research Covers: March 1, 1988; February 15, 1993
Chemical Research in Toxicology Covers: June 1998, July 2007, February 2011
Cancer Epidemiology Biomarkers & Prevention Cover, December 2003
Outstanding Investigator Grant, National Cancer Institute, 1987-2001
Research Career Development Award, National Cancer Institute, 1975-1980
National Research Council Fellow, 1971-1973
Phi Beta Kappa, 1964

Current Research Interests

- Mechanisms and prevention of tobacco-induced cancer
- Carcinogen biomarkers and their application in molecular epidemiology and cancer prevention
- Mechanisms of chemical carcinogenesis in humans
- Chemoprevention of cancer

Selected Active Grant Support

Principal Investigator
Continually funded by the U.S. National Cancer Institute since 1975

- NCI, CA-81301, Metabolism of Carcinogenic Tobacco-Specific Nitrosamines, 1999-
- NCI, CA-203851, e-Cigarettes: Formaldehyde DNA Adducts, Oxidative Damage, and Potential Toxicity and Carcinogenesis, 2017-
- NCI, CA-222005, Clinical Trial of Watercress in Detoxification of Environmental Toxicants and Carcinogens, 2018 -
- NCI, CA-138338 (P01), Mechanisms of Ethnic/Racial Differences in Lung Cancer due to Cigarette Smoking, 2010 -

Co-Principal Investigator
NIEHS, U2CES26533, Minnesota CHEAR Exposure Assessment Hub

Selected Professional Activities

Peer Review
AACR-Johnson & Johnson Lung Cancer Innovation Science Grants Committee, 2017-2019
NIH Center for Scientific Review Special Emphasis Panel, Member 2020; Chair, 2019
NIH Cancer Prevention Study Section, ad hoc, 2018
Special Emphasis Panel, NCI PREVENT Cancer Program, 2011 –
NIEHS Childrens' Health Exposure Analysis Resource Access Committee, 2017 -
Special Emphasis Panel, NCI SPORE grants, 2015
Council for Extramural Grants, American Cancer Society, 2010-2014
Chair, Chemo/Dietary Prevention Study Section, National Institutes of Health 2006-2009
Board of Scientific Counselors, Subcommittee 2, Basic Sciences, National Cancer Institute, 2001-2004
Peer Review Committee on Carcinogenesis, Nutrition, and the Environment, American Cancer Society, 1998-2001; Chair, 2001
Grants Review Panel, American Institute for Cancer Research, 1984-1987
Chemical Pathology Study Section, National Institutes of Health, 1981-1985

Ad Hoc Reviewer:
National Cancer Institute, Cancer Center Support Grant Program

National Institute of Environmental Health Sciences
Dutch Cancer Society
Florida Department of Health
Alberta Heritage Foundation for Medical Research
Veterans Administration
New Jersey Commission on Cancer Research
United States - Israel Bi-national Science Foundation
California Tobacco Related Disease Research Program
Ohio Cancer Research Associates

Selected Advisory Groups and Related Activities

European Food Safety Authority, Contamination Working Group on N-Nitrosamines in Food, 2021
National Research Council Committee on Health Effects and Patterns of Use of Premium Cigars, 2021
U.S. Food and Drug Administration Panel on N-Nitrosamines in Pharmaceutical Products, 2021
Panel Member, 2018 American Cancer Society Professors' Meeting Discussion: "Bad luck" hypothesis
Member (ad hoc), Tobacco Products Scientific Advisory Committee, FDA, 2018
Reviewer, U.S. National Academies, Public Health Risks and Benefits of e-Cigarettes, 2017
Nomination Committee, Division of Chemical Toxicology, American Chemical Society, 2017-2019
Expert Consultation on the Integrated Exposure-Response Function, Univ. of Washington, 2017
Data Safety and Monitoring Board: NHLBI HAPIN study, Household Air Pollution and Health, 2017-
Chair, Nominating Committee, American Chemical Society Sosnovsky Award for Cancer Research, 2014
International Agency for Research on Cancer Monographs Program, Peer Review Committee, 2014
Frontiers in Cancer Prevention Annual Meeting, Program Committee, 2013
Round Table Meeting of the Senate Commission on Food Safety of the German Research Foundation: Nitrate and Nitrite in the Diet, Bonn, Germany, 2012
International Agency for Research on Cancer, Workshops on Tumor Concordance and Meshansims of Carcinogenesis, Lyon, France, 2012
Institute of Medicine, Committee on Scientific Standards for Studies on Reduced Risk Tobacco Products, 2011
AACR Cancer Prevention Committee and Cancer Prevention Summit, 2016
Tobacco Constituents Subcommittee, TPSAC, U.S. Food and Drug Administration, 2010
Flavor and Extract Manufacturers Association Expert Panel, 2010-
AACR Task Force on Tobacco and Cancer, 2009- 2012
External Advisory Board, University of Illinois Cancer Center, 2010-2014
Advisory Committee, Translational Cancer Research Center, South Dakota State University, 2009-2014
Chair-Elect to Past Chair, Chemistry in Cancer Research Working Group, AACR, 2007-2009
Chair, Program Committee, AACR Conference, Chemistry in Cancer Research: A Vital Partnership, 2007
Member, NCI-SRNT FDA Tobacco Regulation Legislation Review Project, 2009
International Agency for Research on Cancer, Knowledge Synthesis in Gene-Environment Interactions in Cancer, Lyon, France, 2009
Strategic Dialogue on Tobacco Harm Reduction, 2006-2007
Committee on Defining Upper Limits for Tobacco Toxicants, WHO TobReg, 2006-2007
Chair, Scientific Advisory Board, Center for Excellence in Environmental Toxicology, University of Pennsylvania, 2005-2010
Chemistry in Cancer Research, AACR, Think Tank of Leaders in the Field, 2005
Chapter Editor for Cancer, Surgeon General's Report, How Cigarette Smoking Causes Disease, 2010
Contributor, Surgeon General's Report, Passive Smoking and Health, 2004; Health Consequences of Smoking, Fifty Years of Progress, 2014
Co-organizer, Symposium on Tobacco Carcinogenesis, American Chemical Society National Meeting, 2005
Program Committee Co-Chairperson, AACR Frontiers in Cancer Prevention Meeting, 2004, 2007

National Cancer Institute Carcinogenesis Think Tank, 2004
National Cancer Institute Biotechnology Initiative for Cancer Public Health Working Group, 2004
National Tobacco Monitoring, Research, and Evaluation Workshop, 2002
International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 37, *Tobacco Habits Other than Smoking*, 1985; Vol. 83, *Tobacco Smoke and Involuntary Smoking*, 2002; Vol. 85, *Betel Quid and Areca Nut*, Chair, 2003; Vol. 89, *Smokeless Tobacco and Some Related Nitrosamines,* 2004; Vol 100E, *A Review of Human Carcinogens-Lifestyle Factors*, 2009
International Agency for Research on Cancer Handbooks on Cancer Prevention, Vol. 9, *Cruciferous Vegetables, Isothiocyanates, and Indole-3-carbinol*, 2003
Lung Cancer Progress Review Group, Co-Chair, Chemoprevention Section, National Cancer Institute, 2001
Board of Scientific Counselors, National Toxicology Program, 1997-2001
Science Advisory Board, National Center for Toxicological Research, FDA, 1998-2002
Board of Scientific Counselors, Division of Cancer Etiology, National Cancer Institute, 1989-1995
Division of Chemical Toxicology, American Chemical Society, Chair, 1999-2000; Chair-elect, 1997-1998; Program Chair, 1996; Chair, Nominations Committee, 2011
Board of Directors, Minnesota Smoke Free Coalition, 1997-2001
Health Research Committee, Health Effects Institute, 1992-1996
External Scientific Advisory Board, Ohio State University Comprehensive Cancer Center, 2002-2006
Corporation Visiting Committee, Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, 2000-2003
External Advisory Committee, Environmental Health Sciences Center, Oregon State University, 1996-2000
Cancer Prevention Think Tank, American Cancer Society, 1995
American Association for Cancer Research Program Committee, 1983, 1990, 1993, 1997, 1999, 2000, 2003-2005, 2009 (co-chair), 2010; Session Chair, 1984, 1986, 1988, 1991, 200, 2003
Advisory Group, Center in Molecular Toxicology, Vanderbilt University School of Medicine, 1991-1997; Chair, 1995-1997
Advisory Panel, Inhalation Toxicology Research Institute, 1990-1996
Advisory Panels, Chemical Industry Institute of Toxicology, 1990-1996
Advisory Panel, NYU-Nelson Institute of Environmental Medicine, 1992-1995
Peer Review Committee-Scientific Council, International Agency for Research on Cancer, 1991
Upper Aerodigestive Cancer Working Group, National Cancer Institute, 1986-1989
Contributor, Surgeon General's Report on the Health Consequences of Using Smokeless Tobacco, 1986

Editorial Activities

Editor-in-Chief, *Chemical Research in Toxicology*, 2013 - 2017
Associate Editor, *Journal of Medicinal Chemistry*, 2004 - 2012
Associate Editor, *Nicotine and Tobacco Research*, 2009 - present

Editorial Boards:

Mutagenesis, 2014 - present
Cancer Research, 1980 - 2000; 2010 - 2012
Cancer Epidemiology, Biomarkers, and Prevention, 1990 - present
Molecular Cancer Therapeutics, 2001 - 2012
Cancer Prevention Research, 2008 – present
Journal of Environmental Science and Health, Part C, 2016 - present
Chemical Research in Toxicology, 1988 - 1990, 1992 - 1994, 2010 - 2012
Lung Cancer, 2001 - 2012
Cancer Letters, 1999 - 2006
Carcinogenesis, 1986 - 1990; 2001 - 2006

Chemico-Biological Interactions, 1992 - 1998
Mutation Research, 2002 - 2007
Clinical Cancer Research, 2007 - 2011

Selected Invited Lectures and Conferences, 2002-2019

Cancer Research Campaign, Manchester, England
State University of New York, Stony Brook
Society of Toxicology National Meetings
New York University
Virginia Piper Cancer Research Institute
Vanderbilt University
Reducing Tobacco Harm Conference, Washington, DC
Diet and Optimum Health, Portland, OR
American Cancer Society, Atlanta, GA
Mechanisms of Carcinogenesis and Xenobiotic Metabolism, Rutgers University
International Symposium on Polycyclic Aromatic Compounds
EMS Special Conference, Breast Cancer and Environmental Mutagens
Mayo Clinic, Rochester, MN
Biomarkers for Tobacco Exposure, Minneapolis
University of Wisconsin
Ohio State University
National Cancer Institute Chemoprevention Branch
Columbia University
Society for Research on Nicotine and Tobacco
East-West Conference on Tobacco and Alcohol
Tobacco Harm Reduction Network
Chemistry in Cancer Research
National Cancer Institute – Frederick
Evaluation of Smokeless Tobacco, Washington, DC
University of California, San Diego
AACR Frontiers in Cancer Prevention Meetings
AACR National Meetings
Society for Research on Nicotine and Tobacco
University of North Carolina
Hormel Institute
University of Pittsburgh
National Cancer Institute – Causes of Cancer
National Cancer Institute – Methods and Biomarkers
Roswell Park Cancer Center
Hanna Symposium, Univ. of Minnesota
New Jersey Governor's Conference on Cancer Prevention
American Chemical Society National Meetings
Dietary Factors and Cancer Prevention, Rochester, MN
Wadsworth Center, Albany, NY
University of Pennsylvania
University of Iowa
University of Louisville
University of Kentucky
3M Company, St. Paul, MN
Reducing Tobacco Use in Minnesota
Penn State, Hershey Medical Center
Northwestern University
MD Anderson Cancer Center (2)
University of Utah
Abbott Laboratories
Virginia Commonwealth University
Medical University of South Carolina
Environmental Mutagen Society, Puerto Rico
Dartmouth University
Toxicology Forum, Washington, DC
Tulane University
Indiana University
South Dakota State University
EOHSI, Rutgers University/UMDNJ
World Conference on Tobacco or Health, Mumbai
International Agency for Research on Cancer, Lyon
Ohio State University
University of Arizona Cancer Center
University of Oklahoma
UCLA Molecular Toxicology
University of Tennessee
Microsomes and Drug Oxidation, Beijing
University of Sao Paulo, Brazil
ETH, Zurich
Biomarkers Workshop, Műnster, Germany
Medical College of Wisconsin
Healthy Foods, Healthy Lives Symposium, Univ. of Minnesota
Japan Society of Clinical Oncology, Yokohama
Nitrate and Nitrosamines, Bonn, Germany
Gordon Research Conference Drug Metabolism, Keynote Speaker
Brown University
University of Rhode Island

Minnesota Department of Health
Beijing University of Technology
Peking University
National Center for Nanoscience and Technology, Beijing
U.S. Food and Drug Administration-e-Cigarettes
North Dakota State University
U.S. Food and Drug Administration-Biomarkers
Joint AACR/IASLC Meeting, San Diego
ETH, Zurich
IASLC Meeting, Vienna, Austria
University of Pittsburgh
Penn State Cancer Institute
King's College, London
American Association for Dental Research
Minnesota Department of Health
Kaohsiung Medical University, Taiwan

University Activities

Principal Lecturer and Organizer

Chemical Carcinogenesis and Chemoprevention, 3 credits, 1998 - 2003

Lecturer

Chemical Aspects of Drug Metabolism and Bioactivation
Advanced Pharmacology
Cancer Epidemiology
Molecular Epidemiology

Academic Program Memberships

Medicinal Chemistry Graduate Program
Pharmacology Graduate Program
Combined M.D./Ph.D. Program

Committees

Masonic Cancer Center: Executive Committee and Cancer Prevention and Control Steering Committee, 1998-2014
Masonic Cancer Center Space Committee, 2016 -
M.D./Ph.D. Program Steering Committee, 2000 - 2009

Memberships

American Association for Cancer Research
American Association for the Advancement of Science
American Chemical Society
American Society of Preventive Oncology
American Society for Mass Spectrometry
International Society for the Study of Xenobiotics
Society for Research on Nicotine and Tobacco
American Society for Pharmacology and Experimental Therapeutics

Selected Contributions to Science (with key references)

1. *Tobacco-specific nitrosamines: identification in tobacco products, carcinogenicity, metabolism, DNA binding, and biomarkers.* The tobacco-specific nitrosamines *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are considered to be important causes of tobacco-induced cancer. We carried out most of the carcinogenicity, metabolism, and DNA binding studies of NNN

and NNK, leading to a broad understanding of their uptake and metabolism in humans. We developed highly sensitive mass spectrometric methods for analysis of their metabolites in humans; the NNAL biomarker in particular has been widely used in multiple studies of tobacco-specific carcinogen exposure and risk for cancer. Our studies on NNAL in the urine of non-smokers exposed to secondhand smoke contributed to the clean indoor air now enjoyed nearly universally.

a. **Hecht, S. S.**, Carmella, S. G., Murphy, S. E., Akerkar, S., Brunnemann, K. D., and Hoffmann, D. (1993) A tobacco-specific lung carcinogen in the urine of men exposed to cigarette smoke. *N. Engl. J. Med.* 329, 1543-1546.

b. **Hecht, S. S.** (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol.* 11, 559-603.

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d. Li, Y., and **Hecht, S. S.** (2021) Identification of an *N*′-nitrosonornicotine-specific deoxyadenosine adduct in rat liver and lung DNA. *Chem. Res. Toxicol.* 34, 992-1003.

2. *Application of tobacco carcinogen and toxicant biomarkers in clinical and epidemiologic studies*. We developed a panel of urinary tobacco carcinogen and toxicant biomarkers, using state of the art high throughput liquid chromatography-mass spectrometric techniques, and have applied these methods in collaborative studies to explore human exposure and risk. Using samples from nested case-control studies within prospective cohorts, we demonstrated that NNAL, nicotine metabolites, and phenanthrene tetraol (a PAH metabolite) were significantly related to lung cancer and that NNN was significantly related to esophageal cancer. We further showed significant differences in levels of these metabolites in ethnic groups with differing risks for lung cancer, and have analyzed more than 60,000 urine samples for multiple biomarkers in a clinical study of the reduced nicotine cigarette.

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3. *Metabolism and DNA adducts of PAH and aldehydes*. We carried out extensive studies on metabolism and DNA adduct formation by these compounds. The results of these studies were consistent with, expanded, and supported the bay region diol epoxide model of PAH carcinogenicity, leading us to develop the phenanthrene tetraol biomarker of PAH exposure plus metabolic activation, and to use high resolution mass spectrometry for analysis of benzo[*a*]pyrene-DNA adducts in the human lung. Our studies on

nitrosamine metabolism evolved to investigations of related metabolically formed aldehydes. Our group was the first to identify acrolein and crotonaldehyde-derived DNA adducts that have been extensively investigated, and we developed the first methods for reliable quantitation of formaldehyde and acetaldehyde-DNA adducts in humans. The latter are particularly relevant to alcohol consumption and its role in carcinogenesis.

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b. Villalta, P. W., Hochalter, J. B., and **Hecht, S. S.** (2017) Ultrasensitive high-resolution mass spectrometric analysis of a DNA adduct of the carcinogen benzo[*a*]pyrene in human lung. *Anal. Chem.* 89, 12735-12742. PMCID: PMC6027747.

c. Yang, J., Balbo, S., Villalta, P. W., and **Hecht, S. S.** (2019) Analysis of acrolein-derived 1,N^2-propanodeoxyguanosine adducts in human lung DNA from smokers and nonsmokers. *Chem. Res. Toxicol.* 32, 318-325. PMCID: PMC6644703

d. Chen, M., Carmella, S. G., Li, Y., Zhao, Y., and **Hecht, S. S.** (2020) Resolution and quantitation of mercapturic acids derived from crotonaldehyde, methacrolein, and methyl vinyl ketone in the urine of smokers and nonsmokers. *Chem. Res. Toxicol.* 33, 669-677. PMCID: PMC7193944

4. *Chemoprevention of cancer*. We applied our understanding of mechanisms of tobacco carcinogenesis to the identification of potential naturally occurring agents which could diminish the risk for cancer. This led to extensive studies on a variety of agents including isothiocyanates, indole-3-carbinol, *myo*-inositol, and related compounds. 2-Phenethyl isothiocyanate (PEITC), a potent inhibitor of carcinogenesis in several systems, was chosen for further development because of its natural occurrence and favorable preclinical profile. Together with our colleagues, we carried out an FDA-approved clinical trial of PEITC as an inhibitor of the metabolic activation of NNK in smokers, which showed modest inhibition, but a far greater effect on detoxification of common environmental agents such as benzene, a lead we are pursuing actively in a clinical trial of watercress, an abundant source of PEITC, to enhance detoxification of these agents.

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5. *Expertise in tobacco carcinogenesis*. I have served on multiple U.S. and W.H.O. committees evaluating the tobacco and cancer problem and recommending solutions, and have regularly contributed to U.S. Surgeon General Reports on tobacco and cancer. I have written numerous invited reviews and book chapters on

aspects of tobacco carcinogenesis. With Professor D. Hatsukami, I am currently editing a book entitled "Tobacco and Cancer: the Science and the Story" to be published in 2021 by World Scientific Press.

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d. **Hecht, S. S.** (2017) Oral cell DNA adducts as potential biomarkers for lung cancer susceptibility in cigarette smokers. *Chem Res Toxicol* 30, 367-375. PMCID: PMC5310195

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Chapters, Invited Articles, Books and Other Papers

1. Hoffmann, D., Schmeltz, I., Hecht, S.S., and Wynder, E.L. Chemical studies on tobacco smoke. XXXIX. On the identification of carcinogens, tumor promoters, and cocarcinogens in tobacco smoke. In: *Smoking and Health: I. Modifying the risk for the smoker*. (E.L. Wynder, D. Hoffmann, and G.B. Gori, eds.), Proc. Third World Conf. on Smoking and Health, Washington, DC:U.S. Govt. Printing Office, 1976, 125-145.
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EXHIBIT 2
Documents Reviewed

ZHP Documents

1. ZHP00007221, Deviation Investigation Report regarding a Suspect Genotoxic Impurity of Valsartan, DCE-18001.
2. ZHP00004352, Deviation Report Form, Q/ZHH QA-079-1.
3. ZHP00013245, Deviation Investigation Report regarding a Suspect Genotoxic Impurity of Valsartan, DCE-18001.
4. ZHP00021297, Customer complaint handling record, Q/ZHH QA-021-4.
5. ZHP00021301.
6. ZHP00021302, May 22, 2018 Email from Xaviar Tang to Yinhua Tang and Others Regarding "Unknown Peaks."
7. ZHP00021305, May 31, 2018 Email from Xaviar Tang to Kevin O'Mahony Regarding "Unknown Peaks."
8. ZHP00021306, June 4, 2018 Email from Xaviar Tang to Jannine Quinn and Kevin O'Mahony Regarding "Unknown Peaks."
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10. ZHP00021308, July 20, 2018 Email from Xaviar Tang to Kevin O'Mahony and Others Regarding "NDMA Impurity."
11. ZHP00021309, July 20, 2018 Letter from Minda Cai to "Whom it may concern" Regarding "NDMA Impurity."
12. ZHP00021310, Inventory Information Collection Form for Huahai Valsartan API.
13. PRINSTON00034943, 1.12.4 Request for Comments and Advice.
14. PRINSTON00036665, Testing Result of N-Nitrosodimethylamine (NDMA).
15. PRINSTON0073338, Module: 3.2.S.2.6 Manufacturing Process Development, Valsartan, USP (Process II).
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17. PRINSTON00020500, 3.2.P.4.1 Specifications of Magnesium Stearate, ANDA 204821.
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1. Lance R. Molnar Deposition Transcript for May 7, 2021.
2. Sanjay Singh Deposition Transcripts for May 20-21, 2021.
3. Ambati Rama Mohana Rao Deposition Transcript for April 30, 2021.
4. Daniel A. Snider Deposition Transcript for March 31, 2021.
5. Richard Derek Glover Deposition Transcripts for March 12 and April 16, 2021.
6. Antony Gomas Deposition Transcript for April 9, 2021.
7. Walt Owens Deposition Transcript for April 21, 2021.
8. Michelle Osmian Deposition Transcript for May 6, 2021.
9. Claire Lyons Deposition Transcript for April 27, 2021.
10. Daniel Barreto Deposition Transcript for April 14, 2021.
11. Jocelyn D. Rivera Deposition Transcript for February 22, 2021.
12. Reddy Neravetla Deposition Transcript for May 26, 2021.
13. Sushil Jaiswal Deposition Transcript for June 4, 2021.
14. Dawn Chitty Deposition Transcript for May 13, 2021.

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EXHIBIT 3

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Exhibit 26

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October 31, 2022

Adam M. Slater
Mazie Slater Katz & Freeman
103 Eisenhower Parkway
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Dear Mr. Slater:

This report is a supplement to my report dated July 6, 2021, providing further discussion of the failure by ZHP to conduct a reasonable risk assessment of chemical reactions and necessary testing with regard to the TEA with sodium nitrite quenching process, and Zinc Chloride process, resulting in the manufacture and sale of valsartan API and finished dose contaminated with NDMA and NDEA. All opinions are stated to a reasonable degree of scientific certainty.

In summary, ZHP (and its subsidiary Shanghai Syncores that developed the zinc chloride process in the laboratory) could have and should have identified the risk of formation of nitrosamines including NDMA and NDEA, and utilized that information to test for and identify, and then prevent the nitrosamine impurities in the valsartan API and finished dose sold by ZHP. This could have and should have been done during and after development of the processes, and throughout the time that ZHP manufactured and sold the contaminated valsartan with those processes.

As stated in my July 6, 2021 report, the processes were flawed from the outset because of the inclusion of chemical reactions that could foreseeably create nitrosamines in the API. Specifically, quenching the sodium azide with sodium nitrite (nitrous acid) in the presence of the product, which led to a reaction between foreseeably created secondary amines and the nitrous acid to create NDMA/NDEA. For example, the 1978 IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, established that the reactions forming nitrosamines including NDMA and the use of mass spectrometry to identify nitrosamines were well known. In this connection, Min Li confirmed that the reaction described in the IARC monograph, "the reaction of dimethylamine hydrochloride with sodium nitrite at an acidic pH yields NDMA," is what occurred in the zinc chloride process, and this chemical reaction was known since 1865. (Min Li 4/21/21 Dep. Tr. 458:13-465:11).

In addition, ZHP has acknowledged the likely occurrence of cross-contamination of valsartan API manufactured with the zinc chloride and TEA with sodium nitrite quenching processes on shared production lines. "During the period when multiple processes co-existed in Workshop 2 and Workshop W02, equipment were cleaned as per corresponding cleaning procedure to control the residue of active substance from the previous batch when switch from one process to another. However, the residual NDMA and NDEA in the equipment after cleaning for process switch were not analyzed…Based on the analysis of the NDMA and NDEA data, the original equipment cleaning procedure applied might not be able to get rid of the NDMA and NDEA residue on the equipment completely." The Report also states that there was a risk of cross-contamination due to solvent recovery for the same reason: ZHP was not looking for NDMA or NDEA because they failed to perform a straightforward assessment of the chemistry. (ZHP Deviation Investigation Report dated November 5, 2018 (DC-18003, PRINSTON0075797, at 126-130). Min Li of ZHP confirmed: "the DEA [diethylamine] is a typical process impurity of TEA, so DEA would also, yeah, would react with the nitrous acid to perform NDEA." With regard to NDMA, "in some of the TEA raw material it may contain a trace amount of, you know, of dimethylamine, okay, so that's one root cause…for some of the, you know, product, they were manufactured, you know, using the share line, you know, with the zinc chloride valsartan." (Min Li 4/20/21 Dep. Tr. 77:8-80:16). Varied NDMA levels were found in the valsartan API produced in the East and West zones at Chuannan, per the TEA process DIR. ZHP identified factors that would impact the NDMA levels. This includes, "number 1, temperature when adding sodium nitrite; number 2, charging speed of hydrochloride acid; number 3, ph control at the end; and number 4, aqueous phased separation time during quenching." In this connection, ZHP recognized that there was "a lack of detailed description in the production processes." ZHP further stated, "Due to the inaccurate description of some of the parameters in the process, there might be likelihood of fluctuation between different workshops or different batches manufactured in the same workshop, which eventually led to the difference in the amount of residual impurities…the residual amounts of NDMA in valsartan API batches." (Peng Dong 4/2/21 Dep. Tr., 536:7-543:2). Assessment and understanding of the potential chemical reactions in each process would have required testing for NDMA and NDEA of each batch of drug product manufactured with both processes, whether due to the process or cross-contamination, and would have shown the presence of NDMA and NDEA in each batch, as applicable.

The readily available scientific knowledge and testing should have been applied to identify the NDMA and NDEA even after the processes were adopted. This should have been apparent to any organic chemist involved in the development or assessment of these processes. Once ZHP went forward with the processes after having failed to detect and prevent the nitrosamine contamination during the development of the processes, ZHP could have and should have identified the nitrosamine impurities before selling the API or finished dose with NDMA/NDEA impurities. The same scientific knowledge and principles I have discussed with regard to the development of the processes was equally available and could and should have been applied when the product was manufactured for sale. This would have been as easy as adding appropriate testing for NDMA and NDEA to the specifications, and

testing each batch of API and finished dose accordingly. The result would have been detection of the nitrosamines.

ZHP has stated that the detection of the nitrosamines was not possible since they had no knowledge of the potential or actual presence of the nitrosamines and did not possess the technological ability to identify these impurities. I disagree. The deposition testimony provides context for this issue. For example:

Jun Du testified with regard to the August 26, 2018 letter written by ZHP (and signed by him) to the FDA, stating in part that, "it is not the residual DMF that reacts with nitrous acid of the next step, but rather it is the trace amount of dimethylamine, an impurity/degradant of DMF that reacts with nitrous acid to form NDMA, which adds a further dimension over the current thinking, logic and strategy for the evaluation of potential genotoxic impurities. It is this extra dimension over the current industry practice that obscured us from foreseeing this impurity during the process change from triethylamine process to zinc chloride process." (Jun Du 5/28/21 Dep. Tr. 232:18-234:6).

In the November 28, 2018 FDA Warning Letter to ZHP, the FDA explicitly, and correctly disagreed with ZHP's position that this could not be known, "You also failed to evaluate the need for additional analytical methods to ensure that unanticipated impurities were appropriately detected and controlled in your valsartan API before you approved the process change….Your response states that predicting NDMA formation during the valsartan manufacturing process required an extra dimension over current industry practice, and that your process development study was adequate. We disagree. We remind you that common industry practice may not always be consistent with cGMP requirements and that you are responsible for the quality of drugs you produce." (ZHP01344159 (ZHP 213)). Dr. Li and Mr. Du agreed with the FDA that ZHP was "responsible for the quality of the drugs" produced by ZHP. (Min Li 4/21/21 Dep. Tr., 426:8-427:5, 430:11-434:10) (Jun Du 5/28/21 Dep. Tr. 247:17-250:22).

The FDA also stated in the Warning Letter, "You are responsible for developing and using suitable methods to detect impurities when developing, and making changes to your manufacturing processes. If new or higher levels of impurities are detected, you should fully evaluate the impurities and take action to ensure the drug is safe for patients." (Jun Du 5/28/21 Dep. Tr. 237:18-243:20). As stated in my prior report, the knowledge, technology, and methods to identify the NDMA and NDEA were readily available and should have been applied to identify the contamination, and this could and should have been done during development of the processes, and then again once ZHP began to manufacture valsartan with those processes for sale.

In this context, a draft of ZHP's deviation investigation report titled "Investigation Regarding an Unknown Impurity (Genotoxic Impurity)" stated that, "Due to insufficient extent and depth of process research at the early stage, as well as insufficient study and understanding of potential genotoxic impurities, only side reaction product and degradation

products were studied, and was unaware of the further reaction between degradation products and raw material." (Min Li 4/22/21 Dep. Tr. 528:5-531:4). This accurately describes the inadequate scientific risk assessment performed by ZHP, since the chemical reactions and means to test for the foreseeable creation of nitrosamines were well known and available. Scientifically reasonable process research, study and understanding of potential genotoxic impurities, would have resulted in recognition of the risk of creating the nitrosamine impurities, and testing that would have demonstrated the presence of these impurities. I know this from my own personal experience utilizing mass spectrometry to identify nitrosamines including NDMA beginning long before development of these processes in 2011, and the scientific literature including what is identified here and in my prior report, as well as in questioning of ZHP witnesses.

The focus on nitrosamines as potential human carcinogens began after the first demonstration of the carcinogenicity of dimethylnitrosamine in 1956 as outlined in my previous reports. The first report of nitrosamine contamination of food was published in 1968, and the first definitive evidence for the presence of dimethylnitrosamine in meat products in 1972.[1] This stimulated the development of reliable analytical methods for nitrosamines, layered on the existing knowledge base. A review published in 1976 notes the initial development of methods for the analysis of trace amounts of nitrosamines: "there is now no doubt that these compounds do occur in trace amounts in various environmental situations."[2] It goes on: "Recently a better standardization of the methodology, using gas-liquid chromatography and mass spectrometry, has yielded more reliable identification of the nitrosamines."

Fine et al reported the development of a highly sensitive and reliable nitrosamine-selective detector (the Thermal Energy Analyser, or TEA) in 1975.[3] Coupling of TEA to gas chromatography (GC-TEA) became the standard method for analysis of ultra-trace levels of nitrosamines. Thousands of products including pharmaceuticals were reliably analyzed and shown to contain trace amounts of nitrosamines (reviewed in Forman, D. and Shuker, D. Nitrate, nitrite and nitroso compounds in human cancer, *Cancer Surveys* 8: 205-487 (1989)), leading to international concern, further analyses, and mitigation efforts. Ultimately with the development of improved gas chromatography-mass spectrometry (GC-MS) methods and the wide availability of this instrumentation by the early 1980s, GC-TEA gave way to GC-MS which was even more reliable because of its ability to directly determine structural information from fragmentation patterns, information that was not available by GC-TEA. A review published in 1989 summarizes hundreds of analyses of nitrosamines in food.[4]

Thus, there is no doubt that the necessary technology and highly reliable methods for the analysis of nitrosamines in various settings were available from the 1970s. More recent analyses have confirmed the earlier data.

The international concern about the presence of these carcinogens in various settings gave rise to the widely attended and recognized International Agency for Research on Cancer conferences on nitrosamines which were held at various locations in the world from 1976-

1991. These meetings produced a series of books describing the research discussed at the meetings.[5]

In summary, nitrosamine contamination of food, drugs, and other products, and the reliable analytical methods to detect nitrosamines, have been known since the 1970s. Routes of formation of nitrosamines under various conditions have been extensively described in numerous publications and textbooks. Chemists using processes which involve the presence of nitrite and secondary amines should absolutely be aware of this huge body of literature, and utilize the widely available technology and methods to identify the nitrosamines resulting from these processes.

ZHP's witnesses acknowledged in their depositions that the chemical reactions were known and that mass spectrometry was available to identify nitrosamines starting before these processes were even developed. Dr. Li ultimately agreed that "the technology and the methodology was clearly available to identify the NDMA," as long as you "know what to look for" based on a risk assessment – which he confirmed is an ongoing process for the lifecycle of the drug. (Min Li 4/20/21 Dep. Tr., 230:9-19, 233:10-18).

Eric Gu also confirmed in his deposition that the 1978 IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans" stated in part: "It has been known since 1865 that the reaction of dimethylamine hydrochloride with sodium nitrite at an acidic pH yields NDMA." (Eric Gu 4/5/21 Dep. Tr., 65:3-65:24). Mr. Gu was shown a 2009 article published in the scientific journal Tetrahedron Letters, titled: DMF, Much More Than a Solvent. The article states that "DMF decomposes slightly at its boiling point to afford dimethylamine and carbon monoxide, this reaction occurring even at room temperature in the presence of acidic or basic materials..." He agreed that DMF could decompose to yield dimethylamine, and this was known in the scientific community. (Eric Gu 4/5/21 Dep. Tr., 172:13-174:9, 183:12-21).

Min Li was shown scientific literature identifying the risk of formation of nitrosamines during his deposition. This includes a textbook first published in 1996 titled, Purification of Laboratory Chemicals, which stated that DMF could decompose at its boiling point to yield dimethylamine. (Min Li 4/21/21 Dep. Tr. 391:13-395:5). Another 2009 scientific article titled "N.N-Dimethylformamide: much more than a solvent," also stated that DMF could decompose to produce dimethylamine, and this article cited a textbook published in 1966. (Min Li 4/21/21 Dep. Tr. 411:19-413:22). In addition, an article published in 2010 by a group from Beijing University of Technology in the Journal of Physical Chemistry titled "Theoretical Investigation of N-Nitrosdimethylamine Formation from Nitrosation of Triethylamine," described the formation of NDMA from the reaction of dimethylamine and nitrous acid. This is what occurred here with the zinc chloride process. (Min Li 4/21/21 Dep. Tr. 414:2-416:12). Dr. Li also stated that this was the reaction that occurred in the zinc chloride process: "the zinc chloride process for the formation of NDMA, you know, was also under the acidic, you know, pH. So, yes, so from that perspective, yeah, they are consistent." He also confirmed that in 2011, "scientists would be aware of and have available to them" this information, as well as

the known availability and use of mass spectrometry to test for potential nitrosamines, as stated in the 1978 Monograph, "The principal techniques employed for the analysis of volatile N-nitrosamines [including NDMA] have been described in a recent publication…The relative merits of high- and low-resolution mass spectrometry are discussed, since use of mass spectrometry as a confirmatory technique is particularly important." (Min Li 4/21/21 Dep. Tr. 458:13-465:11). The literature discussed with the ZHP witnesses provides useful examples and is representative of information that was well known in the scientific literature and scientific community prior to and after the 2011 development of these processes.

The identification of the NDMA and NDEA would have been straightforward to anyone who was familiar with the chemical reactions in the manufacturing process, utilizing mass spectrometry. The location of the NDMA peak found on the chromatograms for the zinc chloride process has been identified by ZHP. For example, Min Li testified that there was a "little peak after the toluene peak" and stated, "And then in the sample injection, this peak turns out, if I remember correctly, to be n-butyl acetate, okay? So that's the peak - - that's the peak, you know, eluting after the toluene peak. Okay. So NDMA would elute on the shoulder, or sometimes may even completely co-elute with this peak." (Min Li 4/20/21 Dep. Tr. 25:16-28:22.). In addition, Qiangming Li confirmed that "[w]hen we used GC-FID for the testing, regarding the peak that appeared after toluene, the response of NDMA was pretty low." (Qiangming Li 4/14/2021 Dep. Tr. 168:17-20). The point is that taking into account the potential creation of nitrosamines should have led to the use of the GC-MS technology to identify the NDMA and NDEA.

A series of customer complaints was received by ZHP with regard to the unknown, or aberrant peaks on the chromatography. This included:

1. Ranbaxy/SunPharma on September 30, 2014 (Qiangming Li 4/14/2021 Dep. Tr. 130:7-170:11; ZHP01748896 (ZHP 260)).

2. Shanghai Pharmtech on November 20, 2014 (*Id.* at 177:22-199:20; ZHP01748905 (ZHP 264)).

3. SunPharma on November 17, 2016 (Qiangming Li 4/15/2021 Dep. Tr. 290:16-318:10; ZHP00405069 (ZHP 277); ZHP01313866 (ZHP 278)).

4. Vertex on December 21, 2016 (Qiangming Li 4/14/2021 Dep. Tr. 204:11-214:17; ZHP02630924 (ZHP 265); ZHP02630926 (ZHP 266).

5. Glenmark on December 29, 2016 (Qiangming Li 4/15/2021 Dep. Tr. 254:22-290:4; ZHP00496153 (ZHP 271); ZHP00496155 (ZHP 272); ZHP02118712 (ZHP 273)).

6. Aurobindo on August 23, 2017 (*Id.* at 343:21-372:9; ZHP02094739 (ZHP 281)).

7. Novartis on May 22, 2018 (*Id.* at 386:17-466:17; ZHP00405021 (ZHP 284)).

Testing with the available technology would have identified the NDMA and NDEA at every point during the period when these processes were used to manufacture the valsartan. Novartis did what ZHP should have done. Novartis investigated the unknown peaks to determine what was causing them, and identified the NDMA in ZHP API manufactured with the Zinc Chloride process. Of note, Novartis inquired of ZHP as to whether DMF was utilized in the zinc chloride process on June 7, 2018, as part of its investigation. (ZHP01390017). This was relevant information to be taken into account by anyone assessing the cause of the unknown peaks since dimethylamine was the degradation/decomposition product of DMF that then reacted with the nitrous acid to form NDMA. ZHP simply ignored or didn't understand this basic chemistry. ZHP failed to perform the same analysis despite knowing the details of the manufacturing process, and this illustrates the inadequacy of ZHP's risk assessment from the perspective of organic chemistry.

I have reviewed chromatograms for the zinc chloride process. The NDMA peak would not have been identifiable as NDMA on the gas chromatography alone, but as stated above if ZHP had been diligent and conducted a scientifically reasonable assessment, they would have recognized the need to test for NDMA, and they could have used the available technology to identify the NDMA peak. We have examples of the results that would have been obtained in the documentation of the testing performed after the disclosure of the NDMA in June, 2018. The September 1, 2018 ZHP Response to DMF Information Request Letter provides a series of chromatograms showing the methods used, and the identification of the NDMA peak. (ZHP00079913). There was nothing complex or difficult about what was done once they were looking for the NDMA (and ultimately NDEA). In another example, the July 20, 2018 Deviation Investigation Report titled: Investigation regarding a Suspected Genotoxic Impurity of Valsartan (ZHP00004363) contains images of the June 6, 2018 email and attachments from Kevin O'Mahony to Xavier and others at ZHP. The chromatograms show the NDMA peak, and the method used to identify the peak, (ZHP00004399-4402). This should have been identified from the outset and at every other point moving forward, including when Novartis and other customers submitted complaints and inquiries regarding unknown peaks, as listed above. In this context, the European authority documented that Novartis had shared its analytical method with ZHP in July, 2017, in rebutting ZHP's argument that it did not have that information until June 2018. (ZHP01862681 (ZHP 232)). Of note, and perhaps not a coincidence, the July 27, 2017 email written by Jinsheng Lin, Ph.D. confirming that there was NDMA in ZHP's valsartan API, caused by the quenching with sodium nitrite, was written during the same month.

The same analysis applies to the NDEA in the valsartan. For example, August, 2018 testing performed by ZHP shows the NDEA peak identified. (ZHP02733180).

When asked why Novartis discovered that an unknown peak was due to NDMA before ZHP, he acknowledged that ZHP was required to investigate the peak, but could not give an explanation, "it was not so easy to detect" and "it's quite a challenging work." (Eric Gu 4/5/21

Dep. Tr., 210:24-219:5, 236:24-237:8). As set forth above, identification was quite feasible and should have been accomplished from the start of development of these processes, through the entire time that the drug products were manufactured and sold. This could have been done at any point, and seeming to contradict ZHP's position that it did not know, the July 27, 2017 email accurately describes the presence of the NDMA and the root cause of quenching with sodium nitrite.

Mr. Gu was questioned about the aberrant/unknown peaks. He had no reasonable explanation for why, despite every batch demonstrating the "NDMA peak just after the Toluene peak on the chromatograms. . . nobody at ZHP realized that it needed to be tested and identified." Mr. Gu admitted that ZHP was aware of these peaks and "did whatever they can," however, "They are struggling, I guess, in the past." (Eric Gu 4/6/21 Dep. Tr., 333:21-335:19). Mr. Gu was not aware that ZHP customer Sun Pharmaceuticals complained of unknown peaks in November 2016, and was not aware that, according to the European Medicines Agency, ZHP did not directly compare the unknown peaks observed by Novartis to ZHP's own gas chromatography. Nor was he aware that Novartis had shared its GC-FID method for evaluating chromatogram peaks with ZHP in July 2017. (Eric Gu 4/5/21 Dep. Tr., 240:3-243:18).

To be clear, the pathway to identification of the NDMA and NDEA impurities continued to be straightforward after the valsartan containing NDMA and NDEA began to be marketed. ZHP could have and should have taken the steps described above from the time they began to sell the valsartan containing NDMA and NDEA until it was discovered by Novartis, with the aid of an outside laboratory in June 2018. The necessary information and technology was readily available the entire time.

In addition to the ease in detecting the NDMA and NDEA with available testing, if ZHP still determined to go forward with these processes, the simple step of extracting the product prior to the quenching could have been taken to prevent the NDMA (and NDEA in the TEA with sodium nitrite process) formed in the zinc chloride process during quenching of the sodium azide from contaminating the drug product. ZHP stated in one document that, "any formation of NDMA will not be carried over into the product," and, "This approach can be done without any change of manufacturing process." July 1, 2018 Investigation of the Source of this Impurity (NDMA) (ZHP01495188). ZHP also provided a detailed analysis at pages 29-35 of 236 of the November 5, 2018 Deviation Investigation Report (PRINSTON0075797), indicating: "After optimization, the ROS remains the same, the product in Valsartan Crude Step (Step 4) is separated before the addition of NaNO2 (and the subsequent addition of Hcl)…Therefore, the product in the organic phase has no chance to be contaminated by NDMA." This would not have changed the manufacturing process for the drug product or route of synthesis as recognized by ZHP, and would not have negatively impacted or introduced any risk to the identity, quality, purity, strength, or stability of the drug products, since the drug product would have been separated from and not been exposed to contamination by the genotoxic impurities created during the quenching step. The same could have been done with the TEA with sodium nitrite quenching process. In the alternative,

ZHP could simply have gone back to the original process that did not involve sodium nitrite quenching, as "no NDMA or NDEA will be formed in Tin process." (November 5, 2018 Deviation Investigation Report, at 68 of 236, PRINSTON0075870).

Eric Gu confirmed that ZHP modified the zinc chloride manufacturing process after the FDA became aware of the NDMA, and agreed that ZHP's, "solution was to quench the azide separate from the product so it wouldn't become contaminated with the NDMA," and, "GC-MS would be used to evaluate all peaks to make sure that they were not genotoxic impurities that needed to be controlled out of the product." (Eric Gu 4/6/21 Dep. Tr., 455:1-458:15). If the solvents presenting the risk of secondary amines and sodium nitrite quenching were to be used, this would have prevented contamination of the drug product, and this testing would have confirmed the lack of NDMA or NDEA; this was absolutely feasible and could and should have been done from development through the entire course of the manufacturing of the drug product if the same solvents and chemicals were to be used in the process.

The ZHP API and Finished Dose Nitrosamine Levels
Are Materially the Same and All Exceed the FDA Levels

Minli Zhang—ZHP's Director of Finished Dose Formulation Quality—testified that ZHP determined its APIs' nitrosamines carried over to the finished dose. (3/26/2021 Minli Zhang Dep. Tr. 509:15-17, 518:18-519:3). Ms. Zhang explained:

> In our investigation report, we compared the NDMA level in the API and the NDMA level in the finished dose products, and we found the results basically matched each other. Therefore, we decided not to test the NDMA level in the finished dose products anymore.
>
> We could simply calculate based on the NDMA level in the API, as well as the amount of API used, to come up with a probable level of NDMA in the finished dose products.

(*Id.* at 521:8-19). This is the chart from the deviation investigation report:

In order to qualify the impurity relationship between the dosage form and API, some batches of API and corresponding dosage form were choose at random to test this impurity by Quality Research Department (QR), the testing result is as below:

表 1：制剂成品及对应 API 批次检测结果列表

Table 1: testing result between dosage form batches and corresponding API

序号 SN	产品名称 Product Name	产品批号 Batch No.	产品规格 Strength (mg)	API 厂家批号 Vendor batch No. Of API	API 结果 Result for API	制剂结果 Result for dosage form
					NDMA 含量(ppm) Assay of NDMA (ppm)	
1.	缬沙坦片 USP Valsartan Tablets USP	341A18007	40	C5523-17-382	81.4	83.1
2.	缬沙坦片 USP Valsartan Tablets USP	342B17012	80	C5523-17-190	101.9	101.0
				C5523-17-191	101.7	
3.	缬沙坦片 USP Valsartan Tablets USP	343G17002	160	C5355-17-132	120.0	110.3
				C5355-17-133	104.5	
4.	缬沙坦片 USP Valsartan Tablets USP	344B17071	320	C5355-17-131	119.3	123.2
				C5355-17-132	120.0	
5.	缬沙坦氢氯噻嗪片 USP Valsartan HCTZ Tablets USP	609B18003	80/25	D5191-16-133	3.4	2.9
6.	缬沙坦氢氯噻嗪片 USP Valsartan HCTZ Tablets USP	611B17003	320/25	D5191-16-027	27.7	31.3
7.	缬沙坦氢氯噻嗪片 USP Valsartan HCTZ Tablets USP	611B17007	320/25	D5191-15-149	7.9	6.4

从上表数据分析，制剂产品与 API 的检测结果的差值接近(0.5~9.7ppm)。

Based on analysis above, the testing difference value of API and dosage form is almost the same (0.5-9.7ppm)

(ZHP00683571, 683578). As a result, ZHP stopped testing FD and blended the API levels to get the FD ones. (*Id.* at 520:22-523:19, 525:12-22 (discussing ZHP 189)). Hai Wang—the President of Solco—confirmed that the API and FD contained the same levels of nitrosamines. (3/10/2021 Hai Wang Dep. Tr., 116:3-118:23, 144:15-147:1). Prinston explicitly informed the FDA that "[i]t is confirmed that NDMA has been present in Valsartan drug substance (API) batches and carried to the drug product Valsartan," relying on the same test results as shown in the above chart. (PRINSTON00249966, 249967; ZHP00099424, 99441-42). ZHP concluded this analysis applied to NDEA as well. (PRINSTON0075797, 75977 (stating: "According to the previous raw material investigation, i.e. presence of diethylamine impurities in triethylamine hydrochloride, combined with the formation mechanism of NDEA, it should be the nitrosation of diethylamine impurities (in triethylamine hydrochloride) by nitrite to produce NDEA impurities, which is carried over into crude products, and finally remain in valsartan finished products.")).

As set forth in my July 6, 2021 report, testing by Teva and Torrent of its finished dose products manufactured using the ZHP contaminated valsartan API also established that the

levels of NDMA and NDEA all exceeded the limits set by the FDA. (TEVA-MDL2875-00546489 (TEVA 155); TORRENT-MDL2875-00005092; TORRENT-MDL2875-00369262; TORRENT-MDL2875-00072916; TORRENT-MDL2875-00366172).

Conclusion

The unreasonably dangerous contamination of valsartan drug products with NDMA and NDEA was easily avoidable, based on prevailing scientific knowledge and technology that existed before, during, and after the development and then commercial use of the zinc chloride and TEA with sodium nitrite quenching processes. The available knowledge and technology should have been applied to add straightforward testing for NDMA and NDEA of each batch of API and finished dose manufactured using the API manufactured with these processes, which would have revealed the presence of the NDMA and NDEA. The contamination of the drug product could have been prevented by extracting the product before quenching the sodium azide.

Stephen S. Hecht

Stephen S. Hecht, Ph.D.
Wallin Professor of Cancer Prevention
American Cancer Society Professor
American Chemical Society Fellow

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[5] International Agency for Research on Cancer (IARC) Books on Nitrosamine Research (each book, about 500 pages). IARC is a branch of WHO. *Environmental N-Nitroso Compounds: Analysis and Formation*, Vol. 1. (E.A. Walker, P. Bogovski, and L. Griciute, eds.), IARC Scientific Publications, No. 14, Lyon, France: International Agency for Research on Cancer, **1976**; *Environmental Aspects of N-Nitroso Compounds*, Vol. 1. (E.A. Walker, M. Castegnaro, L. Griciute, and R.E. Lyle, eds.), IARC Scientific Publications, No. 19, Lyon, France: International Agency for Research on Cancer, **1978**; *N-Nitroso Compounds: Analysis, Formation and Occurrence*. (E.A.

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EXHIBIT A
Supplemental List of Materials Reviewed

ZHP Documents

1. PRINSTON00249966, August 27, 2018 Letter from Prinston to the FDA regarding ANDA 206083.
2. ZHP02326538 (ZHP 189).
3. ZHP00662283, Draft Investigation regarding an unknown impurity (Genotoxic Impurity) (ZHO 212).
4. ZHP01862672, Final GMP Inspection Report (ZHP 232).
5. ZHP01748896, Email Chain between ZHP and Ranbaxy (ZHP 260).
6. ZHP01748905, Email Chain between ZHP and Shanghai Pharmttech Co. Ltd. (ZHP 264).
7. ZHP02630924, Email Chain regarding Vertex (ZHP 265).
8. ZHP02630926, Chronology regarding Vertex (ZHP 266),
9. ZHP00496153, Email Chain regarding Glenmark (ZHP 271).
10. ZHP00496155, Chronology regarding Glenmark (ZHP 272).
11. ZHP02118712, Email Chain between ZHP and Glenmark (ZHP 273).
12. ZHP00405069, Email Chain between ZHP and Sun Pharmaceutical Industries Ltd. (ZHP 277).
13. ZHP01313866, Chromatograms from Sun Pharmaceutical Industries Ltd. (ZHP 278).
14. ZHP02094739, Email Chain between ZHP and Aurobindo (ZHP 281).
15. ZHP00405021, Email Chain between ZHP and Novartis (ZHP 284).
16. ZHP00099424, Meeting Information Package from Prinston regarding ANDA 204821.
17. ZHP01390017, Email Chain Between ZHP and Novartis.
18. ZHP01495187, Investigation of the Source of this Impurity (NDMA).
19. ZHP01344159, November 29, 2018 Warning Letter from the FDA to ZHP (ZHP 213).
20. ZHP01495186, July 1, 2018 Email Enclosing ZHP01495187, Investigation of the Source of this Impurity (NDMA).
21. ZHP02733180, Chromatogram and Results for NDEA in ZHP's valsartan
22. PRINSTON00002249, 1-2 Annex-3 NDMA for TEA Process by GC-MS
23. ZHP02365339, Valsartan Chromatograms
24. ZHP02364173, NDMA and NDEA test results for all batches of Valsartan in USDMF grade
25. ZHP00011368, Certificate of analysis for D5191-14-157M
26. ZHP00344175, Summary of Unspecified Peaks in Residual Solvents Method of Valsartan
27. ZHP00476862, Valsartan Impurities Profile Analysis Report (ZHP 220)

28. ZHP00021455, Study Report of Unknown Peak in Residual Solvent of Valsartan
29. ZHP01870977, Study Report of Unknown Peak in Residual Solvent of Valsartan
30. ZHP02214602-71, Novartis Documents
31. ZHP02633528-ZHP02633538
32. ZHP00405024-ZHP00405068
33. ZHP00380568-ZHP00380591
34. ZHP01748896-ZHP01748899-ZHP1748899 (ZHP 260)
35. ZHP00405069-ZHP00405070 (ZHP 277)
36. ZHP01320376-ZHP01320392 (ZHP 280)
37. ZHP00405021-ZHP00405023 (ZHP 284)
38. ZHP00359796-ZHP00359822 (ZHP 288)
39. ZHP02135008-ZHP02135025 (ZHP 289)
40. ZHP02173090-ZHP00371269 (ZHP 290)

Torrent Documents

1. TORRENT-MDL2875-00072916, Details of Finished good batches (USA market) manufactured at indrad with Huahai API having old ROS.
2. TORRENT-MDL2875-00366172, Valsartan: Impact assessment of NDMA.
3. TORRENT-MDL2875-00369262, Test Results
4. TORRENT-MDL2875-00005092, Details of Finished good batches manufactured at indrad with Huahai API having old ROS.

Deposition Testimony

1. Minli Zhang Deposition Transcript for March 22-26, 2021.
2. Eric Gu Deposition Transcript for April 5-6, 2021.
3. Qiangming Li Deposition Transcript for April 13-16, 2021.
4. Jun Du Deposition Transcript for May 27,-28, 2021.

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2. Ender, F. & Ceh, L. Occurrence of nitrosamines in foodstuffs for human and animal consumption. Food Cosmet. Toxicol. 6, 569-571, doi:10.1016/0015-6264(68)90292-7 (1968);
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6. Hotchkiss, J. H. Preformed *N*-nitroso compounds in foods and beverages. *Cancer Surv* **8**, 295-321 (1989).
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